

AD \_\_\_\_\_

GRANT NUMBER DAMD17-94-J-4078

TITLE: Genomic Instability at Premalignant and Early Stages of Breast Cancer Development

PRINCIPAL INVESTIGATOR: C. Marcelo Aldaz, M.D.

CONTRACTING ORGANIZATION: The University of Texas  
M.D. Anderson Cancer Center  
Houston, Texas 77030

REPORT DATE: August 1999

TYPE OF REPORT: Final

PREPARED FOR: Commander  
U.S. Army Medical Research and Materiel Command  
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;  
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 4

1

20001013 114

# REPORT DOCUMENTATION PAGE

*Form Approved*  
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)			2. REPORT DATE August 1999		3. REPORT TYPE AND DATES COVERED Final (1 Aug 94 - 31 Jul 99)		
4. TITLE AND SUBTITLE Genomic Instability at Premalignant and Early Stages of Breast Cancer Development			5. FUNDING NUMBERS DAMD17-94-J-4078				
6. AUTHOR(S) C. Marcelo Aldaz, M.D.							
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of Texas M.D. Anderson Cancer Center Houston, Texas 77030			8. PERFORMING ORGANIZATION REPORT NUMBER				
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER				
11. SUPPLEMENTARY NOTES							
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE			
13. ABSTRACT (Maximum 200)  As a continuation of our original allelotypic studies, we have determined that allelic imbalances and losses affecting the chromosome 16q arm constitute early abnormalities in breast carcinogenesis since we observed them in a significant number of preinvasive lesions. The most common region of overlapping allelic losses spans the region 16q23.3 - q24.1, observed affected in 60 - 70% of <i>in situ</i> breast lesions. Positional cloning strategies are currently being employed to clone the target genes for such anomalies. We have defined approximately 300 kb of a homozygous deletion within the region q23.3 - q24.1 of chromosome 16 in a breast cancer cell line. We have mapped to this region a large number previously reported ESTs. Several new cDNA clones were isolated and mapped. We sequenced almost 400,000 DNA base pairs from the region of interest and identified numerous putative exons. We identified and are currently characterizing a gene (WWOXID) that maps to this region. The characterization of this gene as well as other potential targets and determining their role in breast carcinogenesis could lead to development of biomarker tools of diagnostic-prognostic significance.							
14. SUBJECT TERMS Breast Cancer				15. NUMBER OF PAGES 171			
				16. PRICE CODE			
17. SECURITY CLASSIFICATION OF REPORT Unclassified		18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified		19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified		20. LIMITATION OF ABSTRACT Unlimited	

## FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

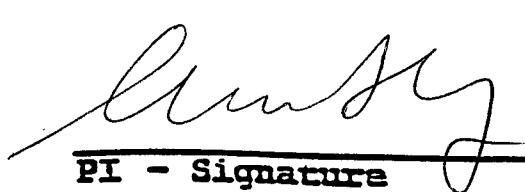
In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

 PI - Signature

8.27.99 Date

**TABLE OF CONTENTS**

Front Cover .....	1
SF298 Report Documentation Page.....	2
Foreword.....	3
Table of Contents.....	4
Introduction .....	5
Body .....	5-15
Key Research Accomplishments .....	15
Reportable Outcomes .....	15-16
Conclusions.....	17
References.....	17-18
Appendix	
Publications	
Abstracts	

## INTRODUCTION

Over the past few years, considerable progress has been accomplished in the elucidation and characterization of genes whose mutation predisposes individuals to risk of developing familial breast cancer. These genes include the recently cloned BRCA1, BRCA2, PTEN and TP53 in the case of Li-Fraumeni syndrome. However, while these genes have been shown to be frequently affected in inheritable forms of breast cancer, there is yet no conclusive evidence to suggest that these genes are also responsible for sporadic breast cancer which accounts for approximately 90-95% of the total breast cancer incidence (reviewed in 1).

Activation of oncogenes (e.g. c-myc, Cyclin D1, ERBB2), alterations in growth factor pathways (e.g. TGF alpha, EGF, IGF and their receptors), inactivation of tumor suppressor genes (e.g. p53, Rb) and chromosomal instability are all commonly found in breast cancers. Although abundant information is available on the mentioned genomic abnormalities in sporadic breast cancer, to date no clear model of the critical events or delineation of primary abnormalities has emerged. Additionally, it is unclear which, if any, of those somatic mutations are causative of breast tumorigenesis. More likely the vast majority of genomic abnormalities described are consequence of tumor progression.

Ductal carcinoma *in situ* (DCIS) of the breast is known as a preinvasive stage of breast cancer and is probably the precursor of infiltrating breast cancer (2). Genetic alterations shown at this stage might indicate association with early events in malignancy or invasiveness. Loss of heterozygosity (LOH) at specific chromosomal loci has been considered as part of the indirect evidence for postulating the existence of possible tumor suppressor genes within those specific chromosome regions. It is known that several mechanisms can lead to the loss of alleles in tumors such as chromosomal deletions, monosomies, mitotic recombination, and unbalanced translocation. Hypothetically, the remaining allele of the tumor suppressor gene in question could be rendered inactive due to events at the gene level such as specific point mutations or other types of inactivating mutations. Usually, LOH at specific chromosome regions affects not only the putative tumor suppressor gene but also neighboring genes or genetic markers that are used as indicators to track down the minimum area of LOH.

Our original hypothesis was that a high level of chromosomal instability already exists at preinvasive stages of breast cancer development. To this end the studies described in the original application focused on early stages of breast cancer development in order to identify the earliest detectable allelic abnormalities correlating with the histological grading and subtype of the lesions. The ultimate goal was to better understand the breast carcinogenesis process and to eventually identify tools of potential diagnostic or prognostic significance.

## BODY

### **Methodology development, DCIS and replication error phenotype studies**

The best obvious source of material for the identification of the various stages of breast cancer progression is available from paraffin-embedded tissues used in routine diagnostic procedures. A first phase of this project consisted in the optimization of a comprehensive technical approach for allowing a multiparametric analysis of human breast cancer lesions from paraffin-embedded tissue

sections. Thus, numerous chromosomal loci can be analyzed from single tissue sections by means of microsatellite length polymorphism analysis. DNA samples from normal and breast cancerous tissue can be obtained from the same section by means of microdissection. This allows to correlate the allelotype of specific lesions with other markers of prognostic and diagnostic significance. The development of such technical approach was reported in a first publication (3), see attached).

As indicated in the introduction is spite of the abundance of data the relevance, role and timing of most of the genetic abnormalities observed in sporadic breast cancer are still unclear. While there is overwhelming evidence that losses of genetic material occur, inherent difficulties exist in determining the relevance of such losses to breast tumorigenesis. In most cases, the tumors analyzed were of the invasive type and/or advanced stages of progression, leading to the question whether these losses are causative factors of tumorigenesis or consequences of the general genomic instability inherent to tumors. Further, it is possible that certain losses may be selected for in the progression or clonal evolution of a tumor to a more advanced type but not necessary for the genesis of the tumor. We hypothesized that some of these questions could be addressed in part through comparative allelotyping of both noninvasive and invasive tumors.

To address the relative timing and frequency of allelic losses of commonly affected regions in breast cancer, microsatellite length polymorphism analysis was performed in a series of preinvasive ductal carcinomas (DCIS) and invasive ductal and lobular carcinomas (4, see attached). Twenty different chromosomal loci were examined in each group. As expected, frequencies of regional losses in invasive ductal carcinomas were similar to those reported by other author (reviewed in 1). However, allelotyping of DCIS samples revealed that chromosomal regions 3p, 3q, 6p, 11p, 16p, 18p, 18q, and 22q were not affected by a high frequency of loss, while analyses of these same regions of invasive tumors showed them to be affected in 10-40% of cases (4). Our findings are in agreement with those of Radford et. al. who examined 61 DCIS samples (5). Because allelic losses affecting these regions were not frequently observed at the noninvasive (DCIS) stage it can be concluded that alterations of these regions are late events in breast cancer progression. More importantly, allelic imbalances observed on chromosome arms 7p, 7q, 16q, 17p, and 17q (4), appear to be early abnormalities because they were observed frequently in DCIS samples.

Lobular carcinomas constitute approximately 10-15% of all breast cancers (2). Histologically, lobular carcinomas have a very distinctive infiltrative growth pattern and metastatic pattern (2). In addition, patients with invasive lobular carcinoma have been reported to have a higher risk of developing multifocal and contralateral breast cancer than those patients with invasive ductal carcinoma (6). To determine whether ductal and lobular carcinomas are subject to the same pattern of allelic loss, comparative allelotyping of the two subtypes was also conducted in our laboratory. Losses of chromosome arms 1p, 3q, 11q, and 18q were more prevalent for invasive ductal carcinoma than for invasive lobular carcinoma (4). However, 8p losses or imbalances were observed in 36% of invasive lobular tumors but only 14% of invasive ductal carcinomas. Interestingly, microsatellite instability was observed in almost 40% of lobular carcinomas, but only 13% of ductal carcinomas (4). This phenomenon of microsatellite instability, also known as RER+ phenotype, is identified by allele size differences between tumor and matching normal controls. First described as a characteristic of tumors from patients carrying an autosomal dominant predisposition to tumors of the colon and endometrium, microsatellite instability has been linked to defects in a group of human mismatch repair genes: hMSH2, hMLH1, hPMS1, and hPMS2 (7-9). Resultant errors in DNA repair are believed to be the cause of the observed genomic instability phenomenon. These data suggest that invasive lobular carcinomas may arise by a mechanism of carcinogenesis different

from that of ductal breast carcinomas and appear to constitute a distinct pathologic entity.

We attempted to extend those earlier observations (4) by analyzing a set of RER+ breast cancer samples for mutations in the DNA mismatch repair gene hMSH2, which as earlier indicated is the most commonly mutated gene in HNPCC kindred.

To this end paraffin embedded tissue sections were obtained from a set of nine breast cancer samples positive for microsatellite instability at multiple loci (more than 5 independent loci). Matching normal control tissues were also obtained from each patient.

Oligonucleotide primers (flanking and nested) for analysis of the 16 HMSH2 exons were synthesized according to the method described by Kolodner et al. (10).

PCR conditions and cycle sequencing were performed according to the same authors (10).

The sequence analysis of the 16 exons of the hMSH2 gene did not show evidence for germinal or somatic mutations. The hMSH2 sequence obtained in all nine RER+ breast cancer cases was normal. (Bednarek A. and Aldaz C. M., unpublished observations).

From this study we concluded that the phenomenon of microsatellite instability observed in a relative small percentage of the sporadic breast cancer cases studied appears to be independent of mutations in the prototypic and most frequently mutated DNA mismatch repair gene hMSH2. Our results are comparable to studies in RER+ sporadic colon cancer cases. Although other DNA mismatch repair genes have not been analyzed these data suggests microsatellite instability in sporadic breast cancer may be the result of a different mechanism and genes to that described in the HNPCC syndrome.

### Chromosome 16 studies

As we earlier mentioned we observed that allelic imbalances and losses affecting chromosome arms 7p, 16q, 17p and 17q appear to be early abnormalities since they were observed in a significant number of DCIS lesions (4).

Due to the paucity of information and the high incidence of allelic losses affecting the long arm of chromosome 16 we decided that it was very important to redirect our efforts and focus in a more in-dept study on the identification of the putative genetic targets for the observed abnormalities. Loss of heterozygosity on chromosome 16q has already been previously reported in breast and prostate cancer with high frequency, indicating the existence of a putative tumor suppressor gene(s) locates in this chromosome arm. We observed that the most commonly affected area spanned the region from marker *D16S515* to marker *D16S504*. Within this region the most affected locus was at *D16S518*, in which LOH was observed in 20 of 26 informative cases (77%). We have estimated that the area of interest lies in subregion q23.3-q24.1. The region of highest LOH spanned approximately 2 Mb, as determined by a yeast artificial chromosome contig covering this region, reported in Chen et al. (11, see attached). Such a high frequency of LOH at a preinvasive stage of breast cancer suggests that a candidate tumor suppressor gene or genes at this location may play an important role in breast carcinogenesis.

To extend these studies we performed a chromosome 16 high resolution allelotyping of a panel of human breast cancer lines in order to identify areas of hemizygosity and homozygous loss (Figure 1). As a natural and important extension of these studies we have built a contig of yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) clones spanning the chromosome 16q region in which frequent allelic losses were detected.

In agreement with our previous findings, most breast cancer lines showed evidence of

hemizygosity affecting all or almost all the chromosome 16q arm. One breast cancer line showed a homozygous loss affecting markers from this area, indicating that a likely target gene for inactivation may reside within this region.

To extend mapping of this deleted area we built a series of new STS markers according to DNA sequences obtained from ends of BAC clones. Using a high density STS map we determined that the specific deletion is approximately 300 kb in size (Figure 2).

In studies in progress we have isolated numerous cDNA clones from a human breast epithelial library that match to this region.

In summary, we have constructed a comprehensive physical map of the region of interest, and we developed the critical resources for the positional cloning of the putative breast cancer suppressor gene.

## Results

The ultimate goal of the ongoing studies is the identification and isolation of genes from chromosome 16 that can play a relevant role early in breast carcinogenesis.

We first defined a relatively small region of homozygous loss (Figure 2) located in area between markers *D16S515* and *D16S504*, that may contain this gene.

We proposed to use standard positional cloning strategies to identify and clone this putative breast cancer suppressor gene.

We characterized a panel of 23 breast cancer cell lines using STS markers as reported in Chen et al. (11). We utilized highly polymorphic markers with high heterozygosity scores (~ 0.70 or more). Given the high polymorphism of the loci investigated, the presence of large areas with lack of heterozygosity in various markers, very likely represent hemizygosity as a consequence of allelic loss. As can be observed in Figure 1, numerous breast cancer lines showed evidence of hemizygosity affecting all or almost all of the chromosome 16q arm. These results were reported (AACR Bednarek and Aldaz). These results are in strong agreement with our previous findings in preinvasive and invasive breast cancer (4, 11). In order to isolate the putative breast cancer suppressor gene residing in the area of interest we built a contig of YAC and BAC clones spanning the target region. Interestingly, we have identified one breast cancer line which showed homozygous losses affecting markers in this region (Figure 2). This indicates that the target gene is very likely contained within this region. Using new STS markers generated from BAC insert ends DNA sequences, we estimated that the homozygously deleted region is approximately 300 kb in length.

In order to better characterize the region of interest, we performed shotgun sequencing of BACs DNA spanning this region. To this end random subclones for sequencing were prepared by DNaseI digestion of the DNA from BACs; 112B17, 249B4, 286F3 and 36O22 (Research Genetics, Inc. CITB-HSP-C library) and fragments of the length approximately 1kbp were cloned into pZERO1 vector (Invitrogen).

We have sequenced so far a total of 382,765 base pairs and the largest contig length is of 96,371 bp, submitted to GeneBank nr division (NCBI, <http://www.ncbi.nlm.nih.gov>) and published under accession number AF179633 (see attached)

In order to isolate cDNAs encoded in this region we are also utilizing a solution hybrid capture method. We have isolated and characterized numerous clones from a human breast cDNA library mapping to two of the BAC clones (249B4 and 286F3) spanning the area of interest. Once the

clones were obtained after sequencing we confirmed mapping by PCR and by hybridization to the BACs DNA. We have isolated 35 cDNAs, 18 new and 17 identified to have high homology to previously known ESTs (est division of GeneBank at NCBI).

Additionally, we are also analyzing, using the BLAST algorithm, the genomic DNA sequenced (382,765 bp) from the area spanning the of homozygous deletion. We have identified additional 32 EST clusters. Using the GRAIL-1.3 algorithm (ORNL, <http://compbio.ornl.gov>), we also performed analysis on the longest contig 96,371 bp to search for putative protein coding regions, CpG islands, RNA polymerase II promoter sites and polyadenylation signal sites. We predicted 32 exons with excellent score and we have been successful to clone cDNAs for six of them.

In summary we analyzed a total of 72 different putative mRNAs mapped to BACs 112B17, 249B4, 286F3 and 36O22. Most of the analyzed cDNAs showed no good open reading frames, ORF and no exon-intron structure when aligned to genomic DNA.

Nevertheless, we identified one cDNA which had exon intron structure. This was determined from partial sequencing of a cDNA clone isolated by the hybrid capture hybridization method and additionally from BLAST identification of one EST (AI219858) which was found to form two putative exons on the genomic DNA sequence (BAC112B17).

Using primers derived from this partial sequence we isolated the whole length cDNA from a human placenta library (RapidScreen cDNA library, OriGene). Additionally, we used 3', 5' RACE on Marathon Ready cDNA library from human mammary gland (Clontech). Both strategies identified the same sequence of this putative gene. The longest clone isolated had a length of 2250bp plus polyA with a 1242bp long open reading frame. Upon further analysis of the existing databases we identified a previously reported partial cDNA sequence of this same gene, 1475 bp long spanning part of the 3' end, this gene has been tentatively defined as a putative oxidoreductase (HHCMA56). Further analysis of EST division of GeneBank returned 30 ESTs with homology to our cDNA clone.

We determined that the identified ORF encodes for a protein 414 aminoacids in length. We used Pfam analysis (<http://pfam.wustl.edu/hmmsearch.shtml>) to search for homology with known protein domains. We observed high homology to two domains; WW domain and short chain dehydrogenase domain. Thus we called this novel protein WWOXID (Figure 3).

The two WW domains are localized on the amino end of WWOXID and the short chain dehydrogenase motif in the central portion of the protein sequence (Figure 3).

### **Expression and mutation analysis**

In order to study expression of this novel gene we performed Northern and RT-PCR analyses on normal human epithelial cells and a panel of breast cancer lines and breast tumors. We used as a probe 1554 bp long EcoRI restriction fragment of clone ORI2 covering 5'UTR and ammoniated coding region and polyA RNA as a target. The length of mRNA according to hybridization was calculated as about 2.2 kbp and correspond with length of the longest cDNA clone. Based on RT-PCR and genomic sequence analyses of the area of interest we also concluded that alternative transcripts may also exist.

As shown in Figure 4, we detected a major transcript of approximately 2.2kbp, only visible (i.e. overexpressed) in some of the breast cancer lines.

We observed much higher expression, compared to normal RNA, of WWOXID in four cancer cell lines, namely; MCF-7, ZR75-1, SKBR3, and UACC812. Hybridization to another breast

cancer cell lines, T47D, MDA-MB453, was slightly higher than to control RNA and to MDA-MB157, MDA-MB435 was on the same level as hybridization to control RNA.

To search for mutations and to map WWOXID on the genomic DNA context we performed identification of exon-intron junctions Figure 5. So far we identified five exons from the 5' end of gene plus we defined the 3' UTR. The physical map of the central region of WWOXID cDNA is currently being further characterized (Figure 5).

We also used a panel of 28 human breast cancer cell lines to search for mutation at the DNA sequence level of WWOXID. So far we have found no evidence of mutations in the first five exons and splicing sites studied from all the cell lines.

The in progress physical mapping of exons and 3'UTR on the genomic sequence show that WWOXID gene spans very likely more than 500 kbp.

### **Putative function of WWOXID**

It is quite difficult to predict the putative role of this protein in the cell but appears to have several interesting features which make it an attractive target for potential abnormalities. The WW domains are known to bind to proline-rich sequences and are usually responsible for protein-protein interactions (12). WW domains were observed in structural, cytoskeletal proteins as well as in enzymes. Some proteins containing WW domain are well characterized. One of them, dystrophin, is found to be responsible, when mutated, for Duchenne muscular dystrophy. Mouse NEDD-4, contains 3 WW motifs, and its role is of a ubiquitin ligase which plays a central role in embryonic development and differentiation of the central nervous system. Another example is the PIN1 protein which is a prolylisomerase involved in cell cycle regulation.

The short chain dehydrogenase domain is a common and very conserved domain found in different oxidoreductase proteins from plants, animals as well as from bacteria. Examples of oxidoreductases include retinol and steroid dehydrogenases which are enzymes participating in the metabolic processing of hormones important for cell function, cell cycle and differentiation.

### **On Going Studies**

#### **Chromosome 16 studies**

We will continue the characterization of the area of interest of chromosome 16. This will include further studies on the potential role of WWOXID in breast cancer as well as the cloning and characterization of additional target genes of interest that might be identified within this chromosome 16 region.

#### **DCIS studies biomarkers of progression**

In collaboration with various investigators from the M.D.Anderson Cancer Center we are pursuing a multiparameter characterization of DCIS. Among the molecular/genetic biomarkers associated with progression of DCIS to invasive breast cancer, we are analyzing the incidence of allelic losses of various of the loci which we determine relevant in our first studies. To this end we are performing a retrospective case-control study of ductal carcinoma in situ with invasive cancer compared with DCIS cases-alone (i.e. with no invasive component).

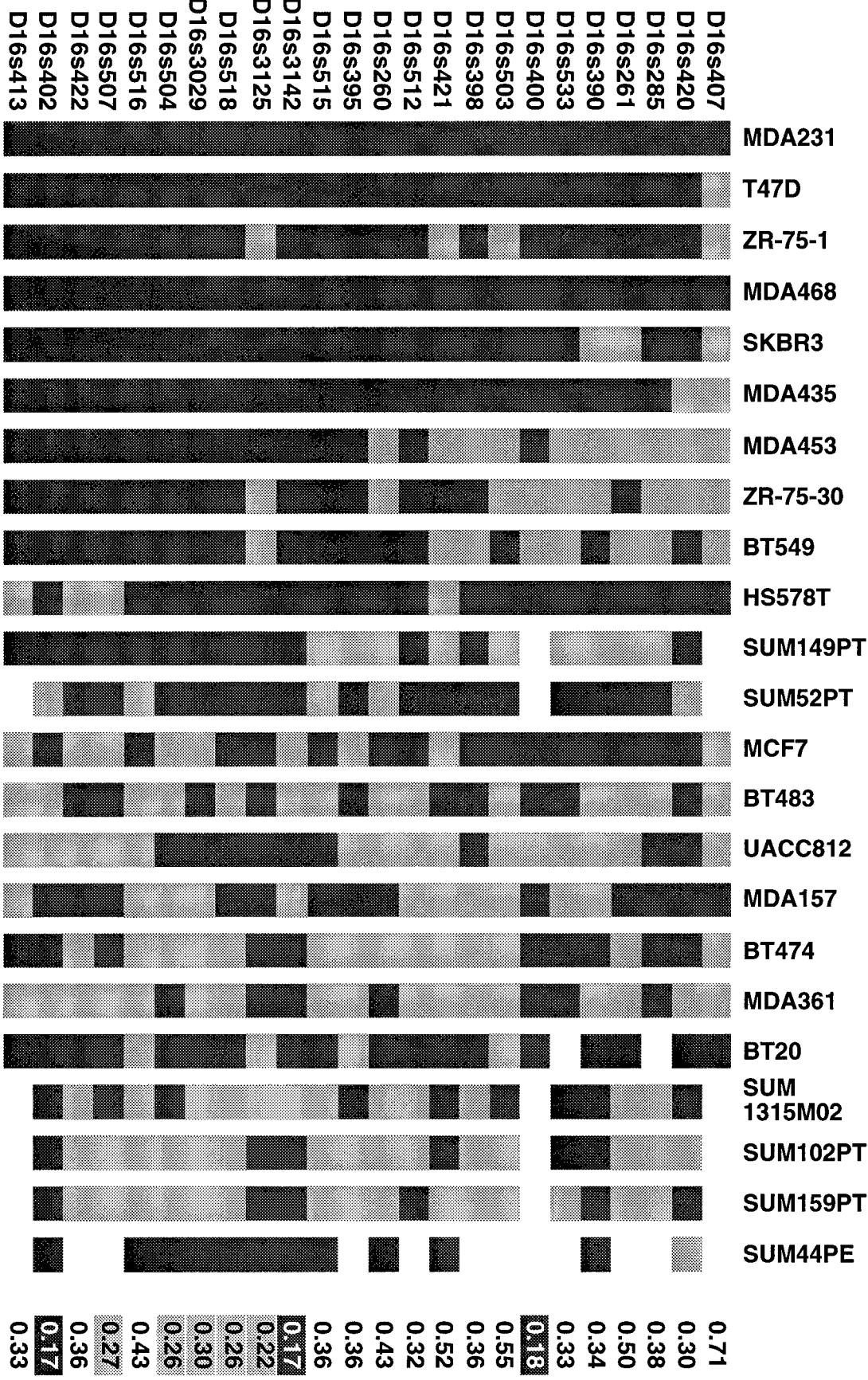


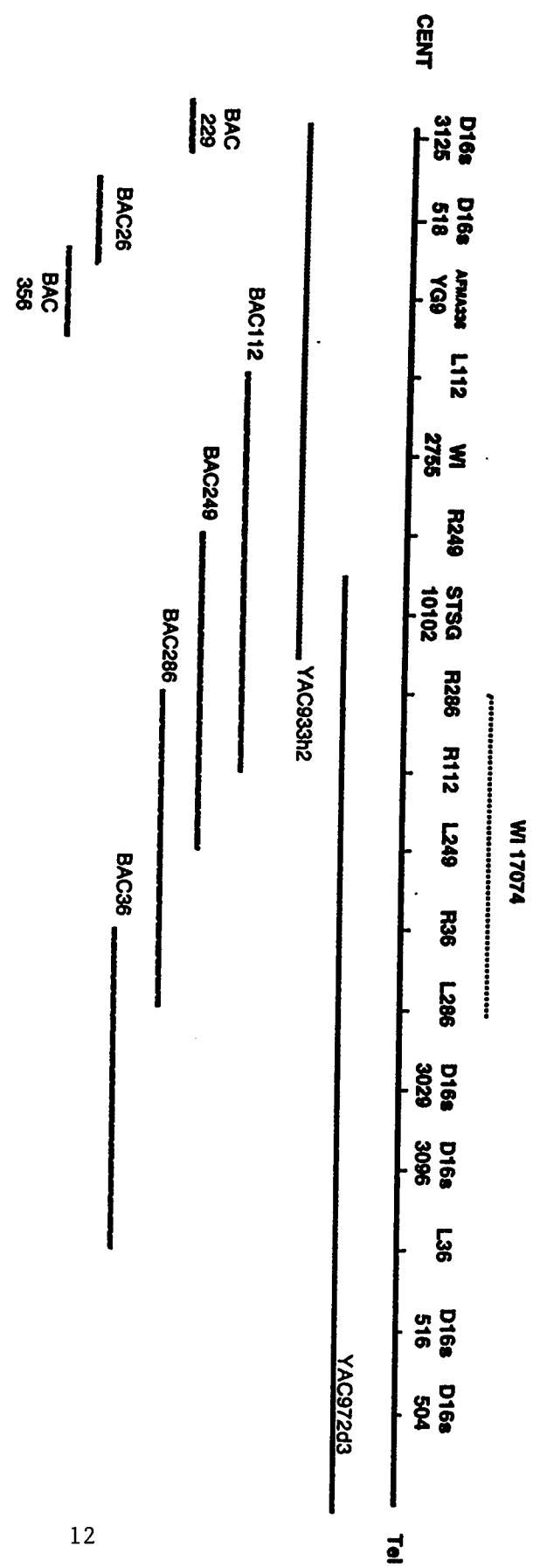
Figure 1

High resolution allelotype of chromosome 16 microsatellite markers in breast cancer cell lines. Markers arranged in mapping and linkage order as previously described in Chen et al. (Cancer Res. 56:5605-5609, 1996)

Dark gray blocks indicate that a single allele was observed at the corresponding locus (i.e. hemi or homozygosity), light gray areas indicates heterozygosity preserved. Blank indicates not done.

Numbers at right represent the heterozygosity scores calculated at each specific locus from analyzing this breast cancer panel.

In agreement with our previous findings, note that several breast cancer lines showed hemizygosity affecting all or most of the chromosome 16q arm.



**Figure 2.**  
YAC and BAC contig spanning a region with observed homozygous loss in one breast cancer line. The various STSs identified and used to built this contig are shown. The represented distance between STSs is not to scale. The homozygous deletion have been defined in the region between STS markers L112 and L36.

A)

1	18	47	59	88	125	330	414
	WW	WW			short chain dehydrogenase		

B)

WW\_rsp5\_WWP: domain 1 of 2, from 18 to 47: score 40.9, E = 1e-09  
 \*->lpsgWeeatdpsGrpiYYvNheTkttgWekP<-\*  
 lp+gWee+t++G + YY Nh ++tgWe+P  
 query 18 LPPGWEERTTKDGWV-YYANHTEEKTQWEHP 47

WW\_rsp5\_WWP: domain 2 of 2, from 59 to 88: score 38.6, E = 4.4e-09  
 \*->lpsgWeeatdpsGrpiYYvNheTkttgWekP<-\*  
 lp gWe+ td++G++ ++v+h++k t++ +P  
 query 59 LPYGWEQETDENQV-FFVDHINKRTTYLDP 88

adh\_short: domain 1 of 1, from 125 to 330: score 89.6, E = 1.8e-24  
 \*->KvaLvtGassGIGlaiAkrLakeGakVvvadrneeklekGavakelk  
 Kv++vTGa sGIG++Ak +a Ga+V++a+rn +++ ++++++  
 query 125 KVVVVTGANSIGFETAKSFALHGAHVILACRNMR--SEA VSRIL 169

query 170 elGgndkdr alaiqlDvtdeesv.aaveqaverlGr1DvLVNNAGgiill  
 e+ k +++a++lD++ sv+ ++e+ +++ +1+vLV+NA  
 EEWH--KAKVEAMTLDLALLRSVqHFAEAFKAKNVPLHVLVCNAA---- 212

query 213 rpgpfaelsrtmeedwdrividvNltgvflltrav1plmamkkrggGrIvN  
 ++ +1+ ++ + +++vN +g f+l++++ + + ++ r+++  
 TFALPWSLT--KDGETTFQVNHLGHFYLVQLLQD--VLCRSAPARVIV 257

query 258 iSSvaGrke.....g.glvvgpggsaYsASKaAvigltrsL  
 +SS + r + +++ ++ + ++ + ++ +++++ aY+ SK i ++ L  
 VSSESHRFTdindslgkldfsrlSpTKNDYWAMLAYNRSKLCNILFSNEL 307

query 308 AlElaphgIrVnavap.GgvdTd<-\*  
 + 1 p+g++ nav+p+ +++ +  
 HRRLLSPRGVTSNAVHPgNMMYSN 330

Figure 3 A) Positions of two WW domains and short chain dehydrogenase domain in WWOXID protein. B) Homology of selected aminoacid sequence from WWOXID(query line) to consensus sequences of WW domain and short chain dehydrogenase domain (top line). Capital letters in consensus line represents conserved aminoacids.



Figure 4 Northern hybridization showing expression of WWOXID in immortalized normal human mammary gland epithelial cells, HME-87 and in various breast cancer cell lines. Target used was poly A RNA; as probe we used a fragment covering 5' UTR and aminoacid coding region.

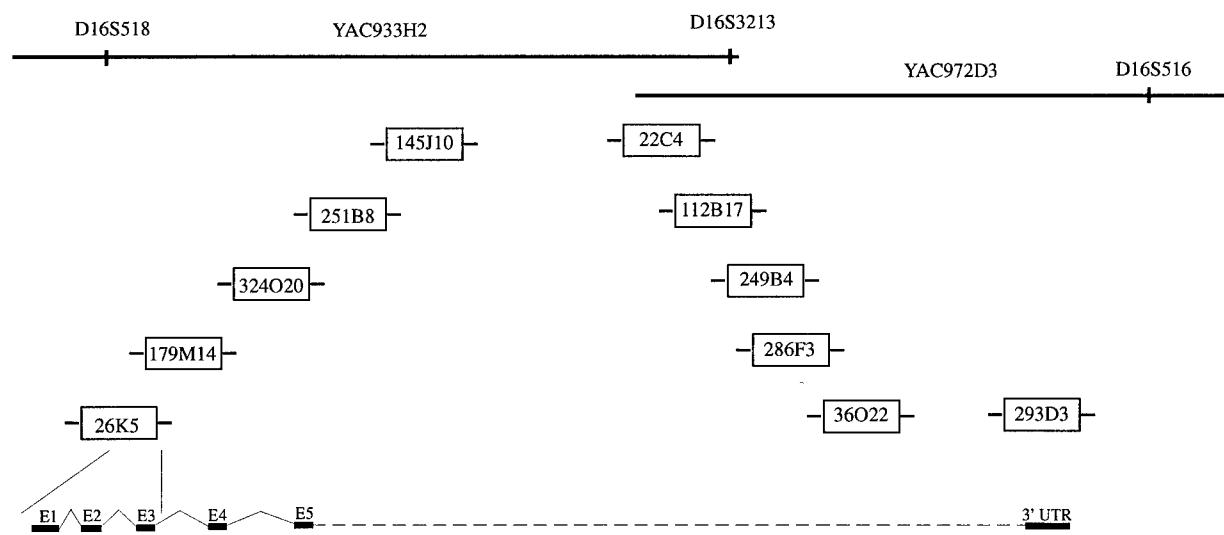


Figure 5 High resolution physical map of chromosome 16q23.3-24.1 region of WWOXID gene. Length of specific BACs and position BAC293D3 are unknown. Total length of chromosome fragment between markers D16S518 and D16S516 is approximately 2Mbp. Contig of BACs 112B7, 249B4, 286F3 and 36O22 according to obtained partial sequence is approximately 400kbp long. E1, E2, E3, E4, E5 and 3'UTR represent positions of known exons and 3' untranslated region of WWOXID in BAC clones.

## KEY RESEARCH ACCOMPLISHMENTS

- 1.** Development of methodology for the analysis of allelic losses in parallel with multiple biomarkers of interest from paraffin embedded microdissected breast premalignant lesions.
- 2.** Identification of the earliest and most common allelic losses observed at preinvasive stages of breast cancer development. (A typical hyperplasias and Ductal Carcinomas *in situ*).
- 3.** Defining and comparing the allelotypic profile of invasive ductal carcinoma and invasive lobular carcinomas.
- 4.** Determining the occurrence of microsatellite instability in a low percentage of breast carcinomas mostly of lobular type.
- 5.** Determining that the occasionally observed microsatellite instability is not due to mutations in the hMSH2 mismatch repair gene.
- 6.** Performing a high resolution allelotyping of chromosome 16 in DCIS lesions and atypical hyperplasias.
- 7.** Identifying and mapping a relatively small specific target area of chromosome 16 commonly lost in breast cancer lines and DCIS lesions.
- 8.** Constructing a YAC and BAC contig spanning the region of interest for positional cloning studies.
- 9.** Sequencing more than 380,000 base pairs of the chromosome 16 area of interest.
- 10.** Cloning of putative target genes from the area of interest such as WWOXID.

## REPORTABLE OUTCOMES

### Publications:

Aldaz, C. M., Chen, T., Sahin, A., Cunningham, J., and Bondy, M. Comparative allelotyping of *in situ* and invasive human breast cancer: High frequency of microsatellite instability in lobular breast carcinomas. *Cancer Res.*, 55: 3976-3981, 1995.

Chen, T., Dhingra, K., Sahin, A., Hortobagyi, G. N., and Aldaz, C. M. Technical approach for the study of the genetic evolution of breast cancer from paraffin embedded tissue sections. *Breast Cancer Res. and Treatment*, 39: 177-185, 1996.

Chen, T., Sahin, A., and Aldaz, C.M. Deletion map of chromosome 16q in ductal carcinoma *in situ* of the breast: refining a putative tumor suppressor gene region. *Cancer Res.* 56: 5605-5609, 1996.

Bednarek, A., Sahin, A., Brenner, A.J., Johnston, D.A., and Aldaz, C.M. Analysis of telomerase activity levels in breast cancer: Positive detection at the *in situ* breast carcinoma stage. *Clinical Cancer Res.* 3: 11-16, 1997.

Brenner, A. and Aldaz, C.M. The Genetics of Sporadic Breast Cancer. *In: Etiology of Breast and Gynecological Cancer*, C.M. Aldaz, Gould, M., J. McLachlan and T.J. Slaga, (eds.) *Progress in Clinical and Biological Research*, Wiley Liss, NY Vol. 396, pp. 63-82, 1997.

Bednarek, A.K., Chen, T., Laflin, K.J., Hawkins, K.A., Liao, Q., and Aldaz, C.M. GeneBank Accession AF179633. , GI: 5823550. Definition Homo sapiens chromosome 16 map 16Q23.3-124.1 sequence. UT MD Anderson Cancer Center, Science Park – Research Division, Smithville, TX 1999

Charpentier, A., and Aldaz, C.M. The Molecular Basis of Breast Carcinogenesis. *In: Molecular Basis of Human Cancer: Genomic Instability and Molecular Mutation in Neoplastic Transformation*. W.B. Coleman and G.J. Tsongalis (eds.) NJ, The Humana Press Inc. in press.

### Abstracts

Chen, T., Dhingra, K., Brenner, A., Sahin, A., Sniege, N., Vogel, V., Hortobagyi, G. N., and Aldaz, C. M. Allelotype of breast carcinoma *in situ* from paraffin sections, correlation with chromosome copy number. *Proc. Amer. Assoc. Cancer Res.*, 35: 118, 1994.

Chen, T., Sahin, A., and Aldaz, C. M. Allelotypic profile and replication error phenotype in lobular vs ductal and *in situ* breast cancers. *Proc. Amer. Assoc. Cancer Res.*, 36: 542, 1995.

Brenner, A.J., and Aldaz, C.M. Chromosome 9p LOH and analysis of MTS1 (p16) in breast cancer. *SABCS*, 1995.

Chen, T., Paladugu, A., Sahin, A., and Aldaz, C.M. Frequent loss of heterozygosity on chromosome 16q in “*in situ*” breast cancer. *Proc. Amer. Assoc. Cancer Res.*, 37: 3771, 1996.

Bednarek, A., Sahin, A., and Aldaz, C.M. Telomerase in human breast cancer. *Proc. Amer. Assoc. Cancer Res.*, 37: 3855, 1996.

Brenner, A.J., Paladugu, A., Dreyling, M.H., Olopade, O.I., Wang, H., Aldaz, C.M. Comprehensive analysis of p16<sup>INK4A</sup> gene and transcripts in breast cancer. *Proc. Amer. Assoc. Cancer Res.*, 37: 4051, 1996.

Cunningham, J.E., Wang, H., Sahin, A., Mastromarino, CL., Bondy, M.L. and Aldaz, C.M. Microsatellite instability as a predictor of second breast cancer. *Proc. Amer. Assoc. Cancer Res.*, 38: 1007, 1997.

Bednarek, A.K. and Aldaz, C. M. Characterization of transcripts from a commonly deleted area of chromosome 16 (q23.3-q24.1) in human breast cancer. *Proc. Amer. Assoc. Cancer Res.*, 39: 872, 1998.

### Personnel

C. Marcelo Aldaz, M.D.	Kendra Laflin
Andrew Bednarek, Ph.D.	Kathleen Hawkins
Taipin Chen	Hui Wang
Melissa Rodriguez	Qiao Yin Liao

## CONCLUSIONS

We were able to demonstrate that chromosomal instability and allelic losses occur early in breast carcinogenesis. We observed the occurrence of such anomalies in Atypical Ductal Hyperplasias and Ductal Carcinoma *in situ* lesions. We defined which anomalies appear to be the earliest and which appear to be the consequence of tumor progression.

Among the abnormalities observed we determined that allelic imbalances and losses affecting the chromosome 16q arm constitute early abnormalities in breast carcinogenesis since we observed them in a significant number of preinvasive lesions. We further defined that the most common region of overlapping allelic losses spans the region 16q23.3 - q24.1, observed affected in 60 - 70% of *in situ* breast lesions. Positional cloning strategies are currently being employed to clone the target genes for such anomalies. We have defined approximately 300 kb of a homozygous deletion within the region q23.3 - q24.1 of chromosome 16 in a breast cancer cell line. We have mapped to this region a large number of previously reported ESTs. Several new cDNA clones were isolated and mapped. We sequence d almost 400,000 DNA base pairs from the region of interest and identified numerous putative exons. We identified and are currently characterizing a gene (WWOXID) that maps to this region. The characterization of this gene as well as other potential targets and determining their role in breast carcinogenesis could lead to development of biomarker tools of diagnostic-prognostic significance.

## REFERENCES

1. Charpentier, A. and Aldaz, C. M. The Molecular Basis of Breast Carcinogenesis. *In: W. B. Coleman and G. J. Tsongalis (eds.), Molecular Basis of Human Cancer: Genomic Instability and Molecular Mutation in Neoplastic Transformation.* New Jersey: The Humana Press, in press.
2. Tavassoli, F. *Pathology of the Breast.* Norwalk, CT: Appleton & Lange, 1992.
3. Chen, T., Dhingra, K., Sahin, A., Sneige, N., Hortobagyi, G., and Aldaz, C. M. Technical approach for the study of the genetic evolution of breast cancer from paraffin-embedded tissue sections. *Breast Cancer Res Treat.* 39: 177-85, 1996.
4. Aldaz, C. M., Chen, T., Sahin, A., Cunningham, J., and Bondy, M. Comparative allelotyping of *in situ* and invasive human breast cancer: high frequency of microsatellite instability in lobular breast carcinomas. *Cancer Res.* 55: 3976-81, 1995.
5. Radford, D., Fair, K., Phillips, N., Ritter, J., Steinbrueck, T., Holt, M., and Donis-Keller, H. Allelotyping of ductal carcinoma *in situ* of the breast: deletion of loci on 8p, 13q, 16q, 17p and 17q. *Cancer Res.* 55: 3399-3405, 1995.
6. Silverstein, M., Lewinsky, B., Waisman, J., Gierson, E., Colburn, W., Senofsky, G., and Gamagami, P. Infiltrating lobular carcinoma. Is it different from infiltrating duct carcinoma? *Cancer.* 73: 1673-1677, 1994.
7. Aaltonen, L., Peltomaki, P., Leach, F., Sistonen, P., Pylkanen, L., Mecklin, J., Jarvinen, H., Powel, I. S., Jen, J., Hamilton, S., and al, e. Clues to the pathogenesis of familial colorectal cancer. *Science.* 260: 812-816, 1993.
8. Fishel, R., Lescoe, M., Rao, M., Copeland, N., Jenkins, N., Garber, J., Kane, M., and

Kolodner, R. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell.* 75: 1027-1038, 1993.

9. Bronner, C., Baker, S., Morrison, P., Warren, G., Smith, L., Lescoe, M., Kane, M., Earabino, C., Lipford, J., Lindblom, A., P. T., Bollag, R., Godwin, A., Ward, D., Nordenskjold, M., Fishel, R., Kolodner, R., and Liskay, R. Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. *Nature.* 368: 258-261, 1994.

10. Kolodner, R., Hall, N., Lipford, J., Kane, M., Rao, M., Morrison, P., Wirth, L., and al, e. Structure of the human MHS2 locus and analysis of two Muir-Torre kindreds for MSH2 mutations. *Genomics.* 24: 516, 1994.

11. Chen, T., Sahin, A., and Aldaz, C. Deletion map of chromosome 16q in ductal carcinoma *in situ* of the breast: refining a putative tumor suppressor gene region. *Cancer Res.* 56: 5605-5609, 1996.

12. Lu, P. J., Zhou, X. Z., Shen, M., and Lu, K. P. Function of WW domains as phosphoserine- or phosphothreonine-binding modules . *Science.* 283: 1325-8, 1999.

## **APPENDIX MATERIAL**

**C. Marcelo Aldaz, M.D.**

**Final Report DAMD17-94-J-4078**

## Comparative Allelotype of *in Situ* and Invasive Human Breast Cancer: High Frequency of Microsatellite Instability in Lobular Breast Carcinomas<sup>1</sup>

C. Marcelo Aldaz,<sup>2</sup> Taiping Chen, Aysegul Sahin, Joan Cunningham, and Melissa Bondy

Department of Carcinogenesis, The University of Texas M. D. Anderson Cancer Center, Science Park, Research Division, Smithville, Texas 78957 [C. M. A., T. C. J., and Departments of Pathology [A. S.] and Epidemiology [M. B., J. C. J.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

### Abstract

To better understand the timing for presentation of allelic losses in human breast carcinogenesis, we compared the allelotypic profile of 23 *in situ* ductal carcinomas with that of 29 invasive ductal carcinomas. We also compared the allelotype of the invasive ductal breast carcinomas with that of 23 invasive lobular breast carcinomas. These studies were performed by means of microsatellite length polymorphisms from microdissected paraffin sections. We observed that involvement of chromosome arms 1p, 3p, 3q, 6p, 16p, 18p, 18q, 22q, and possibly 6q and 11p appear to be late events in breast cancer progression because allelic losses or imbalances affecting these areas were observed with very low frequency at the *in situ* stage. On the other hand, allelic imbalances and losses affecting chromosome arms 7p, 16q, 17p, and 17q appear to be early abnormalities because they were observed in approximately 25–30% of ductal carcinoma *in situ* lesions. Allelic losses and imbalances affecting the 8p arm were frequently observed in invasive lobular breast carcinomas. It was also interesting that microsatellite instability, also known as replication error (RER) phenotype, was found to occur at a high frequency in invasive lobular breast carcinomas because 9 of 23 (39%) were RER+, compared with 7 of 52 (13.5%) RER+ of breast cancers with ductal differentiation ( $P = 0.012$ ,  $\chi^2$  test). Our findings provide for the first time molecular evidence suggesting that invasive lobular breast carcinomas may arise by a different mechanism of carcinogenesis than ductal carcinomas.

### Introduction

Numerous studies have focused on the role of chromosome abnormalities and gene mutations in sporadic breast cancer, but to date no clear model of the critical events or delineation of primary abnormalities has emerged. Various chromosomes or chromosome subregions have been observed to be affected by a high frequency of structural or numerical abnormalities (1). At the molecular level, several somatic mutations have also been described (reviewed in Ref. 2). Amplification or overexpression of several oncogenes, growth factors, and cyclins has been observed at various frequencies (2). Specific allelic losses were also reported at various frequencies, at various chromosome regions, including 1p34–35, 1q23–32, 3p21–25, 6q, 7q31, 11p15, 11q22–23, 13q14, 16q, 17p13, 17q, 18q23–ter and 22q (3–12). Several of these areas appear to be the sites of putative tumor suppressor genes. The tumor suppressor gene *p53* is known to be mutated in a high percentage of breast cancers (13). Despite this abundance of data, the relevance, role, and timing of most of the described genetic abnormalities in sporadic breast cancer are still unclear. It is also not known whether specific mutations play relevant roles as causative

factors or are the consequence of the general genomic instability and progression in breast tumors.

Most of the cytogenetic and molecular information on breast cancer has been obtained by analysis of advanced invasive carcinomas and metastases. In addition, very few studies have discriminated between the different histological types of breast cancer. We therefore focused this study on relatively early stages of breast cancer progression by analyzing preinvasive lesions (DCIS<sup>3</sup>), as well as comparing the allelotype of the two major histological subtypes of invasive carcinomas (*i.e.*, ductal and lobular).

These studies were performed by means of microsatellite length polymorphism analysis of paraffin-embedded tissue sections with simple sequence repeat markers for the chromosome subregions most commonly affected in breast cancer.

### Materials and Methods

Paraffin blocks of breast tumors were randomly chosen from the archives of the Department of Pathology of The University of Texas M. D. Anderson Cancer Center. Five- to 8- $\mu$ m-thick sections were cut from the blocks. The basic technical approach has been described previously (14). Briefly, normal and tumor samples were obtained from different areas of the same section by means of microdissection. After deparaffinization (three washes with xylene for 30 min each), the samples were rehydrated in decreasing concentration of alcohol. DNA was extracted by incubating each sample in 200  $\mu$ l of Instagene chelex matrix solution (Bio-Rad, Hercules, CA) containing 60  $\mu$ g of proteinase K in a shaking incubator at 37°C overnight. Then, the samples were boiled for 10 min, vortexed, and centrifuged at about 7000  $\times$  g for 5 min. Centrifugation produced 150  $\mu$ l of supernatant, of which 2–10  $\mu$ l was used for PCR amplification, depending on the number of cells in the sample. Before PCR, the forward primer was end labeled with T4 polynucleotide kinase (Promega Biotech, Madison, WI) and 6000 Ci/mmol [ $\gamma$ -<sup>32</sup>P]dATP (DuPont New England Nuclear, Boston, MA). PCR was performed in a 20- $\mu$ l reaction volume including 150  $\mu$ M each dNTP, 1 unit of Taq polymerase and 1× Taq buffer (Promega), MgCl<sup>2</sup>, 1 pmol of labeled primer, and 2.5 pmol of unlabeled forward and reverse primers. A “hot-start” procedure was used in which the template and primers were heated in an initial denaturation step of 5 min at 96°C, and cooled to 80°C when the remaining reaction constituents were added, followed by 30–35 cycles at 94°C for 40 s, 55°C for 30 s, and a final elongation step of 72°C for 5 min. The products were electrophoresed on 7% polyacrylamide sequencing gels at 90 W constant power for 2–3 h. The gels were dried at 65–70°C for 1–2 h and exposed to X-ray film for 4 h to overnight. For certain primer sets, the amplification conditions were further optimized by adjusting the MgCl<sup>2</sup> concentration in the reaction buffer. The primers used (Research Genetics, Huntsville, AL) are described in Table 1.

The sample was considered to have partial loss of heterozygosity, or allelic imbalance, if the signal intensity of one allele was diminished by approximately one-half or more of its normal intensity (*i.e.*, in normal tissue) in relation to the remaining allele. Complete loss of heterozygosity was defined as a decrease of 90% or more in the signal intensity of one allele relative to the other.

<sup>3</sup> The abbreviations used are: DCIS, ductal carcinoma *in situ*; RER+, replication error positive; IDCA, invasive ductal carcinoma; ILCA, invasive lobular carcinoma.

Received 6/9/95; accepted 8/4/95.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported by United States Army Breast Cancer Program Grant DAMD 17-94-J-4078 (C. M. A.) and in part by NIH Grant R01 CA59967 (C. M. A.) and a University Cancer Foundation matching supplement.

<sup>2</sup> To whom requests for reprints should be addressed, at University of Texas M. D. Anderson Cancer Center, Department of Carcinogenesis, P.O. Box 389, Smithville, TX 78957.

Table 1 Frequency of allelic losses or imbalances in breast tumors by histology

Marker	Location	No. of tumors affected/no. of informative loci (%)			
		DCIS	IDCA	P value <sup>a</sup>	ILCA
D1S228	1p36-34	1/15 (7)	6/19 (32)	0.07	2/16 (12)
D1S213	1q31-32	3/20 (15)	7/23 (30)	NS	4/18 (22)
D3S1298	3p24.2-22	0/19 (0)	5/23 (22)	0.03	4/17 (24)
D3S1309	3q21.3-25.2	0/11 (0)	4/16 (25)	0.07	1/13 (8)
D6S276	6p22.3-21.3	0/11 (0)	3/10 (30)	0.05	4/10 (40)
D6S255	6q25.2	1/12 (8)	5/19 (26)	NS	4/13 (31)
D7S481	7pter-p15	4/12 (33)	5/16 (31)	NS	5/16 (31)
D7S550	7q36-qter	3/15 (20)	3/14 (21)	NS	1/13 (8)
D8S264	8p21-pter	1/15 (7)	2/14 (14)	NS	5/14 (36)
D8S256	8q24.13-qter	2/12 (17)	1/8 (13)	NS	0/9 (0)
HBB	11p15.4	0/12 (0)	2/14 (14)	NS	3/12 (25)
INT2(FGF3)	11q13.3	2/17 (12)	7/24 (29)	NS	2/16 (13)
D13S155	13q14.3-21.2	2/13 (15)	3/10 (30)	NS	2/8 (25)
D16S407	16p13.13	0/15 (0)	8/20 (40)	0.005	7/16 (44)
D16S413	16q24.3	5/20 (25)	9/21 (43)	NS	6/15 (40)
D17S513	17p13	4/14 (29)	8/14 (57)	NS	6/16 (38)
D17S579	17q12-21.3	6/21 (29)	6/26 (23)	NS	9/16 (52)
D18S59	18pter-p11.22	0/14 (0)	3/13 (23)	0.06	6/15 (40)
D18S51	18q21.33	1/13 (8)	10/23 (44)	0.025	2/12 (17)
D22S283	22q12-13	0/14 (0)	5/14 (36)	0.01	6/17 (35)

<sup>a</sup>  $\chi^2$  test, 1 df, IDCA versus DCIS. NS, not significant.

## Results and Discussion

**DCIS and IDCA Allelotypes.** One goal of this study was to determine which of the chromosome areas most commonly affected by allelic losses or imbalances in breast cancer were involved in the preinvasive stages of breast carcinogenesis. To that end we focused on the chromosome subregions reported to be affected in previous studies (2-12). We selected a panel of representative microsatellite markers mapping to those specific areas (Table 1). It is important to note that allelic losses and in particular allelic imbalances at specific loci do not necessarily imply the presence of a tumor suppressor gene in that area. Duplication of specific chromosome arms can also lead to an allelic imbalance. We view microsatellite length polymorphism analysis as a tool for measuring the general level of genomic instability at specific stages of tumor progression and also for identifying the chromosome arms affected at specific stages of progression. We analyzed with this approach a total of 75 breast cancer samples (23 DCISs, 29 IDCAs, and 23 ILCAs) at 20 different loci. The results obtained from the analysis of invasive ductal carcinomas validates the general approach because we observed similar frequencies of allelic losses to those reported previously (Refs. 2-12; Table 1; Fig. 1). We compared the incidence of allelic losses and imbalances in the DCISs and IDCAs to determine which chromosomal areas are already altered at the carcinoma *in situ* stage and which abnormalities are later events in ductal breast carcinogenesis. Seventeen of the 23 DCIS lesions (74%) had loss or imbalance of at least one locus (*i.e.*, only six of the tumors did not show any abnormality). The results are summarized in Fig. 1 and Table 1. No allelic losses were observed in any DCIS tumor affecting markers from chromosome arms 3p, 3q, 6p, 11p, 16p, 18p, 22q, and low frequency (5-15% of informative cases) for markers from arms 1p, 1q, 6q, 8p, 8q, 11q, 13q, and 18q. From Fig. 1 we can conclude that alterations in chromosome arms 1p, 3p, 3q, 6p, 16p, 18p, 18q, 22q, and possibly 6q and 11p appear to be late events in breast cancer progression because allelic losses or imbalances affecting these areas were not frequently observed at the DCIS stage. We can also conclude that allelic losses or imbalances affecting chromosome arms 16q, 17p, and 17q appear to be early abnormalities because they were observed in approximately 25-30% of DCIS. It was interesting that we also observed a considerable incidence of allelic imbalance affecting marker D7S481, which is on the short arm of chromosome 7, both in DCIS and invasive breast carcinomas (Fig. 1 and Table 1). This chromosome area was not reported previously to be frequently deleted

in breast cancer. However, as mentioned previously, some of the imbalances observed could be the consequence of overrepresentation of chromosome 7p. Representative allelic losses and imbalances affecting the 16q marker D16S413 are shown in Fig. 2A. Loss of alleles on 16q in invasive breast cancer has been reported by several groups (12, 15-17). At least two different regions on 16q have been reported to be involved in allelic loss and to possibly contain tumor suppressor genes (16, 17). Our findings also agree with those of previous cytogenetic studies that also implicated 16q as a possible site for primary chromosomal rearrangements in breast cancer (18, 19).

To our knowledge, this is the first report of a thorough allelotypic analysis of DCIS lesions. Previous reports have focused on the analysis of allelic losses on specific chromosome arms such as 17p (20) and more recently 11q (21). In agreement with the first of those reports (20), we observed 17p losses at the DCIS stage (Ref. 14, Fig. 1). On the other hand, we did not find a high incidence of losses at the DCIS stage (Fig. 1) affecting the INT2 locus on 11q, as reported previously (21). This discrepancy probably is due to the fact that the study of Zhuang *et al.* (21) was performed on microdissected carcinoma *in situ* component of invasive tumors, whereas our study was performed on pure DCIS tumors with no invasive components.

Because DCIS lesions are a heterogeneous group in which the architectural pattern, nuclear grade, and presence of necrosis are thought to be prognostically important (22), the lesions we studied were subclassified according to the presence or absence of necrosis and nuclear grade. They were classified by a nuclear grading system into two groups: high-grade and non-high-grade DCIS. High-grade applied to poorly differentiated tumors and non-high-grade to moderate-to-well differentiated lesions. We also established an index of allelic loss or imbalance for each tumor in which the number of allelic losses or imbalances per tumor was divided by the number of informative loci per tumor. There was no association between the presence or absence of necrosis and the allelic loss index. However, a possible association was observed between allelic imbalance index

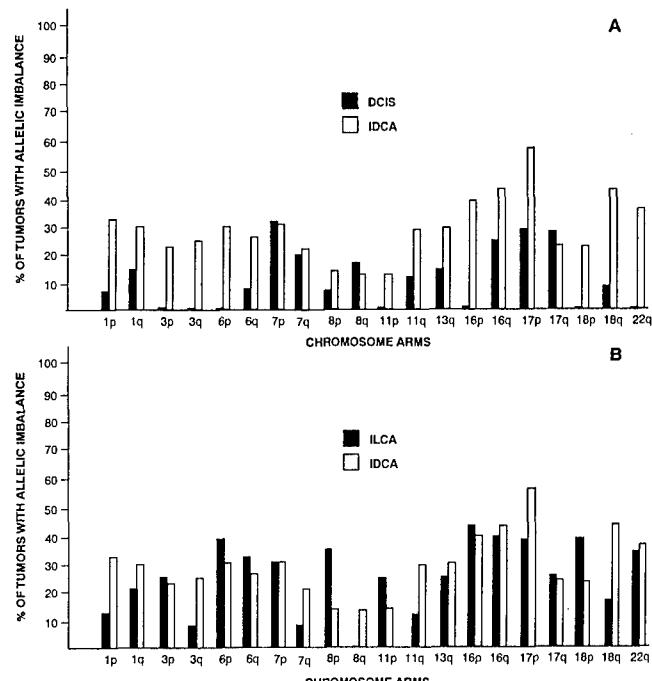


Fig. 1. A, comparative allelotyping of breast DCIS ( $n = 23$ ) versus IDCAs ( $n = 29$ ). B, comparative allelotyping of IDCAs ( $n = 29$ ) with that of ILCAs ( $n = 23$ ).

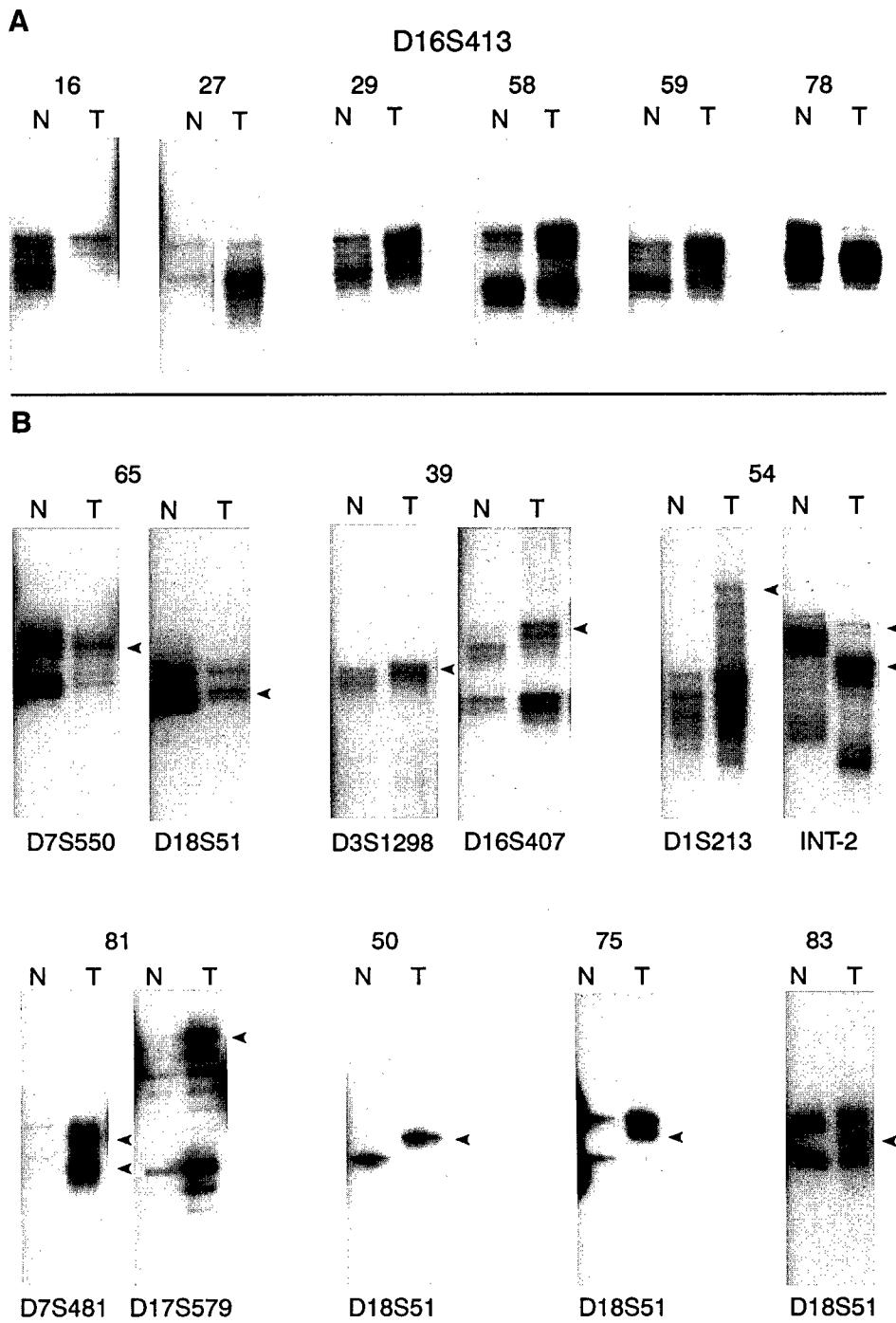


Fig. 2. *A*, representative microsatellite length polymorphism analysis of marker *D16S413* of paired normal (*N*) and breast tumor (*T*) samples obtained from microdissected paraffin-embedded tissue sections. Allelic loss or imbalance was frequently observed affecting this microsatellite marker. Samples 16, 27, 29, and 78 are from IDCAs, and samples 58 and 59 are from DCIS lesions. *B*, various representative breast cancer samples with RER+ phenotype (microsatellite instability). Note the abnormalities in allele size (arrows) in samples from the same tumors at different chromosome loci. Sample 65 is from a DCIS, sample 39 is from an IDC and the rest of the samples are from representative invasive lobular carcinomas. Marker *D18S51*, the only tetranucleotide repeat of the panel used, was frequently affected.

and nuclear grade; lesions classified as non-high-grade (moderate and well differentiated lesions) had overall the lowest indices, with a few exceptions. All six DCIS cases without allelic abnormalities were classified as non-high nuclear grade. All the high-grade DCISs (poorly differentiated tumors) had indices higher than 0.10 (*i.e.*, they had loss or imbalance in at least 10% of the informative markers analyzed). The mean allelic imbalance index for the high nuclear grade DCIS tumors ( $n = 14$ ) was  $0.175 (\pm 0.06)$  and for the non-high grade lesions ( $n = 9$ )  $0.095 (\pm 0.12)$ . Although this putative correlation did not reach statistical significance, probably due to the sample size, it appears that there is a tendency for association between high nuclear grade and higher frequency of allelic losses and imbalances. This is in agreement with previous

histopathological studies that indicated that high nuclear grade appears to identify subsets of DCIS with worse prognosis (22).

**ILCA Allelotype and Microsatellite Instability.** We also compared the allelotypic profiles of invasive ductal carcinomas with ILCA. "Ductal" and "lobular" do not denote a different site of origin; in fact, it has been shown that most of both types of tumors originate in the terminal duct lobular unit (23). However, there are distinct morphological differences between the two histological types. Approximately 10–15% of all breast cancers are ILCA (23). Histologically, lobular carcinomas have a distinctive infiltrative growth pattern with characteristic cytological features (23). As recently reviewed by Silverstein *et al.* (24), reports on the prognosis of ILCA vary considerably. Because characteristically these tumors show diffuse

Table 2 Analysis of breast tumors with microsatellite instability by histology

Histology	RER+ tumors/total tumors
Ductal carcinoma <i>in situ</i>	3/23 (13%)
Invasive ductal carcinoma	4/29 <sup>a</sup> (14%)
	7/52 <sup>b</sup> (13%)
Invasive lobular carcinoma	9/23 <sup>c</sup> (39%)

a versus c;  $P = 0.036$ ,  $\chi^2$  test. b versus c;  $P = 0.012$ ,  $\chi^2$  test.

growth pattern without a prominent mass, they are more difficult to detect and diagnose. It is interesting that patients with ILCAs were reported to have statistically significantly higher risk of subsequent development of contralateral breast carcinoma (24). It has also been observed that the metastatic pattern of infiltrating lobular carcinomas differs from that of invasive ductal carcinomas (25). In our comparative allelotyping of invasive ductal *versus* invasive lobular carcinomas, we observed that allelic losses and imbalances affecting chromosome arms 1p, 3q, 11q, and 18q were more frequent in invasive ductal than in invasive lobular breast cancers (Fig. 1). On the other hand, 8p losses or imbalances were observed in 36% of invasive lobular tumors but in only 14% of invasive ductal tumors (Fig. 1). However, these differences between the two tumor types are not statistically significant at the 0.05 level, and a larger sample is necessary to conclusively identify specific anomalies.

Nevertheless, in the course of the allelotyping studies we observed that numerous lobular tumor samples showed frequent abnormalities in the allele size migration in polyacrylamide gels when compared with the matched normal controls (Fig. 2B). Abnormalities in size of simple sequence nucleotide repeats is a phenomenon described as microsatellite instability (26). This phenomenon has been described as a characteristic of tumors from patients carrying the autosomal dominant predisposition to tumors of the colon and endometrium, known as hereditary nonpolyposis colon cancer (26). These studies led to the identification of a group of human DNA mismatch repair genes as the cause of such general genomic instability phenomenon. Germline mutations in either the *Escherichia coli mutS* homologue *hMSH2* or the *mutL* homologues *hMLH1*, *hPMS1*, and *hPMS2* have been found in different subsets of hereditary nonpolyposis colon cancer kindreds (27, 28). Microsatellite instability, also known as RER phenotype, has also been reported to occur at various frequencies in various sporadic neoplasias other than colon cancer, such as cancers of the endometrium (29), stomach (30), esophagus (31), bladder (32), and other

tissues. Yee *et al.* (33) reported microsatellite instability in 20% of breast cancers. In some other studies, however, a very low frequency of microsatellite instability was detected in breast cancer (34, 35). These discrepancies may be due to the number of loci and the type of simple sequence repeats assayed. For instance, it has been suggested that tetranucleotide repeats are more sensitive to RERs than are dinucleotide repeats (36). Recently, Glebov *et al.* (37) observed that individuals with a family history of breast cancer and with *p53* mutations had a higher frequency of abnormalities of chromosome 17 loci.

In our study of unselected breast cancer cases and mostly dinucleotide repeat markers, we observed the RER+ phenotype in 16 of the 75 breast cancer samples (21%). This figure is similar to that reported by Yee *et al.* (33). It was interesting, however, that when analyzed by histological subtype, only 13% (7 of 52 tumors) of ductal tumors (DCIS plus invasive ductal tumors) showed the RER+ phenotype, in contrast to 39% (9 of 23) of infiltrating lobular breast carcinomas (Table 2). This difference is statistically significant by  $\chi^2$  analysis ( $P = 0.012$ ). Furthermore, if we exclusively compare invasive ductal carcinomas with invasive lobular carcinomas, the difference is still significant ( $P = 0.036$ ). To address whether the observed microsatellite instability could be simply the consequence of a more aggressive tumor phenotype, we plotted the allelic loss index for the DCISs and the invasive ductal and lobular tumors, identifying those samples that were RER+ (data not shown). We observed that the lobular breast carcinomas do not appear to represent a more advanced tumor stage because overall they had a similar level of allelic losses as the invasive ductal tumors. In addition, some tumors with very few losses (low indices) were already RER+, including three at the DCIS stage.

Our data suggest that invasive lobular breast carcinomas appear to arise by a mechanism of carcinogenesis different from that of ductal breast carcinomas and may constitute a possible different pathological entity. These findings also support previous observations of different clinical behaviors of lobular breast tumors and ductal tumors (23–25). As mentioned earlier, the diagnosis of lobular breast carcinoma has been associated with a higher risk for development of multifocal or subsequent contralateral breast cancer (23, 24). The possibility exists that some patients that develop lobular breast tumors could harbor or develop mutations in any of the DNA mismatch repair genes in the mammary epithelium, thus producing a field defect and constituting a

Table 3 Breast cancer patients with microsatellite instability in their tumors

Patient	No. of Loci Affected	Histology	Age	Other cancer <sup>a</sup>	No. of relatives with cancer			
					FDR <sup>b</sup>		SDR	
					Breast	Other	Breast	Other
66	1	Ductal	54		0	2	0	1
5	1	Ductal	43		0	0	2	1
77	1	Ductal	63	Melanoma	0	1	0	2
8	1	Lobular	66	Breast	0	1	0	3
75	1	Lobular	46		0	0	0	0
65	2	Ductal	29		0	0	1	0
37	2	Ductal	66		2	0	1	0
80	3	Lobular	51	Breast, cervix	0	2	0	1
83	3	Lobular	63		0	1	0	2
24	>3	Ductal	49		0	1	1	4
39	>3	Ductal	52		0	0	1	1
50	>3	Lobular	71	Endometrium	0	0	1	3
51	>3	Lobular	73	Breast, endometrium	0	1	0	1
53	>3	Lobular	69	Breast	1	1	0	1
54	>3	Lobular	76		0	2	0	0
81	>3	Lobular	52		1	0	1	2

<sup>a</sup> Other neoplasia in the same patient.

<sup>b</sup> FDR, first-degree relatives (mother, father, siblings, children); SDR, second-degree relatives (aunts, uncles, grandparents, grandchildren).

facilitating event for the development of secondary mutations leading to tumor development.

Liu *et al.* (38) observed that only 1 of 10 patients with RER+ sporadic colorectal cancers had a detectable germline mutation in any of the known DNA mismatch repair genes, and most of the mutations found in the sporadic cases were somatic (38). It is important to analyze the role of microsatellite instability in breast cancer in light of the findings of Glebov *et al.* (37), who reported an association between microsatellite instability and familial history of breast cancer. The samples we used, however, were obtained at random from pathology files, and detailed information on familial history of breast cancer was not available for most of the cases, so we cannot evaluate in detail at this point the association between microsatellite instability and family history. Nevertheless, to at least partially address this point, we conducted telephone interviews with the patients (or their next of kin) who had breast tumors with the RER+ phenotype. We obtained detailed family histories on all first- and second-degree relatives (Table 3). Most of the breast cancers observed in family members, however, were among older relatives, suggesting that these are probably sporadic breast cancers. It was interesting that four of the nine patients with lobular breast cancer and the RER+ phenotype had, previously or synchronously, another breast cancer. In addition, three of these four cases were among those with multiple affected chromosomal loci. On the basis of our observations, we speculate that detection of microsatellite instability has the potential to be useful in identifying patients at risk of developing second breast cancers. However, these are only observations, and these findings will certainly be substantiated with a larger data set.

In summary, in this report we identified for the first time the chromosome arms most frequently affected by losses and imbalances at preinvasive stages of breast carcinogenesis and those allelic losses involved in more advanced stages of progression. In the course of these studies, we also observed that microsatellite instability was much more frequent in infiltrating lobular breast cancers than in ductal breast tumors. Our findings suggest that infiltrating lobular breast carcinoma is a different entity from ductal carcinoma and may arise by a different mechanism of carcinogenesis.

### Acknowledgments

We thank Estelle Kastleman and Phyllis Adatto for conducting most of the telephone interviews for family history information, Judy Ing for artwork, and Michelle Gardiner for secretarial assistance.

### References

- Thompson, F., Emerson, J., Dalton, W., Yang, J-M., McGee, D., Villar, H., Knox, S., Massey, K., Weinstein, R., Bhattacharyya, A., and Trent, J. Clonal chromosome abnormalities in human breast carcinomas. I. Twenty-eight cases with primary disease. *Genes Chromosomes & Cancer*, 7: 185-193, 1993.
- Devilee, P., and Cornelisse, C. J. Somatic genetic changes in human breast cancer. *Biochim. Biophys. Acta*, 1198: 113-130, 1994.
- Genardi, M., Tsihira, H., Anderson, D. E., and Saunders, G. F. Distal deletion of chromosome 1p in ductal carcinoma of the breast. *Am. J. Hum. Genet.*, 45: 73-82, 1992.
- Chen, L. C., Dollbaum, C., and Smith, H. S. Loss of heterozygosity on chromosome 1q in human breast cancer. *Proc. Natl. Acad. Sci. USA*, 86: 7204-7207, 1989.
- Ali, I. U., Lidereau, R., and Callahan, R. Presence of two members of c-erbA receptor gene family (c-erbA and c-erbA2) in smallest region of somatic homozygosity on chromosome 3p21-25 in human breast carcinoma. *J. Natl. Cancer Inst.*, 81: 1815-1920, 1989.
- Deville, P., van Vliet, M., van Sloun, P., Dijkshoorn, K., Hermans, J., Pearson, P., and Cornelisse, C. Allelotype of human breast carcinoma: a second major site for loss of heterozygosity is on chromosome 6q. *Oncogene*, 6: 1705-1711, 1991.
- Bieche, I., Champeme, M. H., Matifas, F., Hacene, K., Callahan, R., and Lidereau, R. Loss of heterozygosity on chromosome 7q and aggressive primary breast cancer. *Lancet*, 339: 137-143, 1992.
- Ali, I., Lidereau, R., Theillet, C., and Callahan, R. Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. *Science (Washington DC)*, 238: 185-188, 1987.
- Carter, S. L., Negrini, M., Baffa, R., Gillum, D. R., Rosenberg, A. L., Schwartz, G. F., and Croce, C. M. Loss of heterozygosity at 11q22-q23 in breast cancer. *Cancer Res.*, 54: 6270-6274, 1994.
- Lundberg, C., Skoog, L., Cavenee, W. K., and Nordenskjold, M. Loss of heterozygosity in human ductal breast tumors indicates a recessive mutation on chromosome 13. *Proc. Natl. Acad. Sci. USA*, 84: 2372-2376, 1987.
- Cropp, C. S., Lidereau, R., Campbell, G., Champeme, M. H., and Callahan, R. Loss of heterozygosity on chromosomes 17 and 18 in breast carcinoma: two additional regions identified. *Proc. Natl. Acad. Sci. USA*, 87: 7737-7741, 1990.
- Sato, T., Tanigami, A., Yamakawa, K., Akiyama, F., Kasumi, F., Sakamoto, G., and Najaam, Y. Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. *Cancer Res.*, 50: 7184-7189, 1990.
- Runnebaum, I. B., Nagarajan, J., Bowman, Soto, D., and Sukumar, S. Mutations in p53 as potential molecular markers for human breast cancer. *Proc. Natl. Acad. Sci. USA*, 88: 10657-10661, 1991.
- Chen, T., Dhingra, K., Sahin, A., Hortobagyi, G. N., and Aldaz, C. M. Technical approach for the study of the genetic evolution of breast cancer from paraffin embedded tissue sections. *Breast Cancer Res. Treat.*, in press, 1995.
- Lindblom, A., Rotstein, S., Skoog, L., Nordenskjold, M., and Larsson, C. Deletions on chromosome 16 in primary familial breast carcinomas are associated with development of distant metastases. *Cancer Res.*, 53: 3707-3711, 1993.
- Cleton-Jansen, A. M., Moerland, E. W., Kuipers-Dijkshoorn, N. J., Callen, D. F., Sutherland, G. R., Hansen, B., Devilee, P., and Cornelisse, C. J. At least two different regions are involved in allelic imbalance on chromosome arm 16q in breast cancer. *Genes Chromosomes & Cancer*, 9: 101-107, 1994.
- Tsuda, H., Callen, D. F., Fukutomi, T., Nakamura, Y., and Hirohashi, S. Allele loss on chromosome 16q24.2-qter occurs frequently in breast cancers irrespectively of differences in phenotype and extent of spread. *Cancer Res.*, 54: 513-517, 1994.
- Dutrillaux, B., Gerbault-Seureau, M., and Zafrani, B. Characterization of chromosomal anomalies in human breast cancer. A comparison of 30 tetraploid cases with few chromosome changes. *Cancer Genet. Cytogenet.*, 49: 203-217, 1990.
- Pandis, N., Heim, S., Bardi, G., Idvall, I., Mandahl, N., and Mitelman, N. Whole-arm (1;16) and i(1q) as sole anomalies identify gain of 1q as a primary chromosomal abnormality in breast cancer. *Genes Chromosomes & Cancer*, 5: 235-238, 1992.
- Radford, D. M., Fair, K., Thompson, A. M., Ritter, J. H., Holt, M., Steinbrueck, T., Wallace, M., Wells Jr., S. A., and Donis-Keller, H. R. Allelic loss on chromosome 17 in ductal carcinoma *in situ* of the breast. *Cancer Res.*, 53: 2947-2950, 1993.
- Zhuang, Z., Merino, M. J., Chuaqui, R., Liotta, L. A., and Emmert-Buck, M. R. Identical allelic loss on chromosome 11q13 in microdissected *in situ* and invasive human breast cancer. *Cancer Res.*, 55: 467-471, 1995.
- Lagios, M. D., Margolin, F. R., Westdahl, P. R., and Rose, M. R. Mammographically detected ductal carcinoma *in situ*. Frequency of local recurrence following tylectomy and prognostic effect of nuclear grade on local recurrence. *Cancer (Phila.)*, 63: 618-624, 1989.
- Tavassoli, F. A. *Pathology of the Breast*, Norwalk, CT: Appleton & Lange, 1992.
- Silverstein, M. J., Lewinsky, B. S., Waisman, J. R., Gierson, E. D., Colburn, W. J., Senofsky, G. M., and Gamagami, P. Infiltrating lobular carcinoma. Is it different from infiltrating duct carcinoma? *Cancer (Phila.)*, 73: 1673-1677, 1994.
- Harris, M., Howell, A., Chrissohou, M., Swindell, R. I. C., Hudson, M., and Sellwood, R. A. A comparison of the metastatic pattern of infiltrating lobular carcinoma and infiltrating duct carcinoma of the breast. *Br. J. Cancer*, 50: 23-30, 1984.
- Altonen, L. A., Peltomäki, P., Leach, F. S., Sistonen, P., Pylkkänen, L., Mecklin, J-P., Järvinen, H., Powell, S. M., Jen, J., Hamilton, S. R., Petersen, G. M., Kinzler, K. W., Vogelstein, B., and de la Chapelle, A. Clues to the pathogenesis of familial colorectal cancer. *Science (Washington DC)*, 260: 812-816, 1993.
- Fishel, R., Lescie, M. K., Rao, M. R. S., Copeland, N. G., Jenkins, N. A., Garber, J., Kane, M., and Kolodner, R. The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis colon cancer. *Cell*, 75: 1027-1038, 1993.
- Bronner, C. E., Baker, S. M., Morrison, P. T., Warren, G., Smith, L. G., Lescie, M. K., Kane, M., Earabino, C., Lipford, J., Lindblom, A., Tannergard, P., Bollag, R. J., Godwin, A. R., Ward, D. C., Nordenskjold, M., Fishel, R., Kolodner, R., and Liskay, R. M. Mutation in the DNA mismatch repair gene homologue *hMLH1* is associated with hereditary non-polyposis colon cancer. *Nature (Lond.)*, 368: 258-261, 1994.
- Risinger, J. I., Berchuck, A., Kohler, M. F., Watson, P., Lynch, H. T., and Boyd, J. Genetic instability of microsatellites in endometrial carcinoma. *Cancer Res.*, 53: 5100-5103, 1993.
- Han, H-J., Yanagisawa, A., Kato, Y., Park, J-G., and Nakamura, Y. Genetic instability in pancreatic cancer and poorly differentiated type of gastric cancer. *Cancer Res.*, 53: 5087-5089, 1993.
- Meltzer, S. J., Yin, J., Manin, B., Rhyu, M-G., Cottrell, J., Hudson, E., Redd, J. L., Krasna, M. J., Abraham, J. M., and Reid, B. J. Microsatellite instability occurs frequently and in both diploid and aneuploid cell populations of Barrett's-associated esophageal adenocarcinomas. *Cancer Res.*, 54: 3379-3382, 1994.
- Gonzalez-Zulueta, M., Ruppert, J. M., Tokino, K., Tsai, Y. C., Spruck III, C. H., Miyao, N., Nichols, P. W., Hermann, G. G., Horn, T., Steven, K., Summerhayes, I. C., Sidransky, D., and Jones, P. A. Microsatellite instability in bladder cancer. *Cancer Res.*, 53: 5620-5623, 1993.
- Yee, C. J., Roodi, N., Verrier, C. S., and Parl, F. F. Microsatellite instability and loss of heterozygosity in breast cancer. *Cancer Res.*, 54: 1641-1644, 1994.
- Wooster, R., Cleton-Jansen, A. M., Collins, N., Mangion, J., Cornelis, R. S., Cooper,

C. S., Gusterson, B. A., Ponder, B. A., von Deimling, A., Wiestler, O. D., Cornelisse, C. J., Devilee, P., and Stratton, M. R. Instability of short tandem repeats (microsatellites) in human cancers. *Nature Genet.*, 6: 152-156, 1994.

35. Lothe, R. A., Peltomäki, P., Meling, G. I., Aaltonen, L. A., Nyström-Lahti, M., Pykkänen, L., Heimdal, K., Andersen, T. I., Moller, P., Rognum, T. O., Fossa, S. D., Haldorsen, T., Langmark, F., Brogger, A., de la Chapelle, A., and Borresen, A-L. Genomic instability in colorectal cancer: relationship to clinicopathological variables and family history. *Cancer Res.*, 53: 5849-5852, 1993.

36. Mao, L., Lee, D. J., Tockman, M. S., Erozan, Y. S., Askin, F., and Sidransky, D. Microsatellite alterations as clonal markers for the detection of human cancer. *Proc. Natl. Acad. Sci. USA*, 91: 9871-9875, 1994.

37. Glebov, O. K., McKenzie, K. E., White, C. A., and Sukumar, S. Frequent *p53* gene mutations and novel alleles in familial breast cancer. *Cancer Res.*, 54: 3703-3709, 1994.

38. Liu, B., Nicolaides, N. C., Markowitz, S., Willson, J. K. V., Parsons, R. E., Jen, J., Papadopoulos, N., Peltomäki, P., Chapelle, A., Hamilton, S. R., Kinzler, K. W., and Vogelstein, B. Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nature Genet.*, 9: 48-55, 1995.

Report

## Technical approach for the study of the genetic evolution of breast cancer from paraffin-embedded tissue sections

Taiping Chen,<sup>1</sup> Kapil Dhingra,<sup>2</sup> Aysegul Sahin,<sup>3</sup> Nour Sneige,<sup>3</sup> Gabriel Hortobagyi<sup>2</sup> and C. Marcelo Aldaz<sup>1</sup>

<sup>1</sup> Department of Carcinogenesis, Science Park – Research Division; <sup>2</sup> Department of Medical Oncology;

<sup>3</sup> Department of Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, USA

**Key words:** breast cancer, allelotype, *in situ* hybridization, paraffin sections

### Summary

We have optimized a technique that allows the study of numerous chromosomal loci ( $n = 20–50$ ) from single paraffin-embedded tissue sections by microsatellite length polymorphism analysis. DNA samples from normal and breast cancerous tissue can be obtained from the same section by means of microdissection. This technique was further improved by subjecting DNA to several cycles of amplification with a degenerate (universal) primer and then with specific microsatellite primers. This amplified DNA was also used to screen for mutations in the p53 gene by means of PCR-SSCP. In addition adjacent tissue sections were used to assess specific chromosome copy number by interphase cytogenetic analyses (chromosome *in situ* hybridization) and to analyze expression of specific genes such as p53 and ERBB2. As an example of the use of our approach we performed a detailed chromosome 17 allelotypic analysis in 22 breast tumors (5 ductal carcinomas *in situ*, 13 invasive ductal carcinomas, and 4 invasive lobular carcinomas). We detected mutations in the p53 gene by PCR-SSCP in 36% of the samples. Samples with significant levels of p53 protein accumulation detected by immunohistochemistry were also positive for mobility shifts in the SSCP analysis. We observed that chromosome 17 allelic losses and imbalance occurred at as early a stage as ductal carcinoma *in situ* (DCIS). Although in some cases we observed allelic losses or imbalance affecting the 17p13 region, close to the p53 locus, several of the tumors showed dissociation between such loss or imbalance and p53 mutation. Lobular carcinomas were predominantly disomic for chromosome 17 in contrast with ductal tumors, which often showed polysomy for chromosome 17. This comprehensive approach correlating the tumor subtype, its allelotype, with specific chromosome copy number and specific gene mutations and expression in preinvasive or early invasive breast cancer lesions will potentially provide information of relevance for a better understanding of the multistep mechanisms of breast carcinogenesis.

### Introduction

Numerous somatic mutations affecting various genes and chromosome regions have been described in human breast cancer. However, the relevance

and role in sporadic breast cancer of most of these abnormalities is still unclear [1–3]. It is very important to determine whether some of these anomalies are cause or effect of tumor progression [2]. Thus, there is a need for studies addressing the

sequentiality and timing of the various genomic abnormalities from the putative breast premalignant lesions to the most aggressive malignant phenotypes. The best obvious source of material for the identification of the various stages of progression is available from paraffin-embedded tissues used in routine diagnostic procedures.

In this report we describe the optimization of a comprehensive technical approach for a multiparametric analysis of human breast cancer lesions from paraffin-embedded tissue sections. By analyzing preinvasive and early invasive breast cancer lesions, this approach allows determination of the timing of presentation of several of the most common genomic abnormalities. The techniques described here allow the analysis of normal and pathological template DNA from microscopic lesions. Our approach is based on the use of microsatellite chromosome markers (simple sequence repeats or SSRs) for tissue allelotyping [4]. SSRs became tools of common use in the analysis of genetic abnormalities in carcinogenesis [5, 6]. The frequent polymorphism in their length among different individuals makes SSRs particularly valuable for the detection of allelic losses or imbalance affecting specific chromosome areas. They also allow the identification of tumors that may be generated due to errors in DNA mismatch repair and characterized by a general microsatellite instability [7].

Interphase cytogenetics chromosomal *in situ* hybridization, or CISH, is another recently developed technique being used extensively for the study of genomic abnormalities in solid tumors [8, 9]. This technique can also be applied to paraffin-embedded tissue sections [10–12]. CISH allows evaluation of the degree of intratumor clonal heterogeneity and eventually identification of tumor subpopulations on microscopic lesions [12]. The optimization of micromolecular techniques such as SSR analysis of chromosomal loci from paraffin-embedded sections, coupled with other techniques in current use such as interphase cytogenetics and conventional immunohistochemistry, will allow valuable retrospective studies of archival tissues to be done.

## Materials and methods

### *Microsatellite analysis*

Five- to eight-micron-thick sections were cut from paraffin-embedded tissue blocks. Tissue microdissection was done on paraffin-embedded sections by drawing the silhouette of the area of interest on an H&E-stained slide and overlapping with the unstained specimen or by deparaffinizing first, staining with toluidine blue, and then microdissecting. Normal and tumor samples can be obtained from different areas of the same section or alternatively samples for normal DNA can be obtained from additional paraffin blocks from unaffected tissues (e.g. lymph nodes). After deparaffinizing (3X xylene/30 min), samples were rehydrated in decreasing alcohol gradients. DNA was extracted by incubating in 200 µl Instagene chelex matrix solution (BioRad) containing 60 µg of proteinase K. Incubations were carried out in a shaking incubator at 37° C overnight. After proteinase K digestion, samples were boiled for 10 min, vortexed, and centrifuged at > 7,000 G for 5 minutes. After centrifugation, 150 µl of usable volume was produced: of this, 2–10 µl were used for PCR amplification, depending upon the cellularity of each sample. Prior to PCR reactions, the forward primer was end labeled with T4 polynucleotide kinase (Promega) and 6,000 Ci/mmol [ $\gamma$ -<sup>32</sup>P] = dATP (NEN). PCR reactions were performed in a 20 µl reaction volume 150 µM each dNTP, 1 u Taq polymerase, 1 X Taq buffer (Promega), –2mM MgCl<sub>2</sub>, 1 pmol labeled primer and 2.5 pmol unlabeled forward and reverse primers.

In a 'hot start' procedure template and primers were heated to 96° C and denatured for 5 min. The remaining reaction constituents were added later at 80° C. The DNA was then subjected to 30–35 cycles of 40 sec at 94° C and 30 sec at 55° C, and a final elongation step of 5 min at 72° C. Products were electrophoresed on a 7% polyacrylamide sequencing gel at 90 w constant power for 2–4 hr. Gels were dried at 65–70° C for 1–2 hr and exposed to X-ray film from 4 hr to overnight. For certain primer sets, the amplification conditions were further optimized by titrating the MgCl<sub>2</sub> concentration in the

reaction buffer. In some cases it may be necessary to use higher annealing temperatures (60° C or 65° C). Primers used were: D17S513 [13], D17S579 [14], MPO [15], D17S784, D17S849 [16], and D17S520 (J. Weber, unpublished data).

An alternative approach was used to analyze extremely small lesions or to generate additional template DNA. This approach was based on the use of a degenerate universal primer (DOP-PCR), as described by Telenius *et al.* [17]. To this end a 1–2  $\mu$ l sample from the original 150  $\mu$ l template-containing solution was obtained. This sample was used as template for one round of PCR amplification with the universal degenerate primer 5'-CCGACTCGAGNNNNNATCTGG-3' [17]. The reaction mixture contains a template DNA sample, 1.5  $\mu$ M universal primer, 200  $\mu$ M each dNTP, 2 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 0.1 mg/ml gelatin, and 2.5 U Taq polymerase in a 50  $\mu$ l reaction volume.

For this alternative approach, reaction mixtures were subjected to one cycle of 4 min at 93° C; 8 cycles of 1 min at 94° C, 1 min at 30° C, and 3 min at 72° C; 28 cycles of 1 min at 94° C, 1 min at 56° C, and 3 min at 72° C, and a final extension at 72° C for 10 min. The resulting PCR product was then used as the template (1  $\mu$ l) for a second PCR using either the specific microsatellite primers as described above or the specific p53 exon primers for PCR-SSCP analysis.

#### PCR-SSCP analysis

PCR-SSCP analysis of exons 5–8 of the p53 gene was performed using a commercially available human p53 amplimer panel (Clontech Lab. Inc.) [18]. Each PCR was done in a 20  $\mu$ l volume containing 3 pmol of each primer at a 1:3 labeled/unlabeled ratio (both primers were previously end labeled with  $\gamma$ -<sup>32</sup>P), 300  $\mu$ M dNTPs, 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01% (weight/volume) gelatin, and 1.0 U Taq DNA polymerase. Samples were overlaid with 25  $\mu$ l of mineral oil and then amplified in 35 cycles of 1 min at 94° C, 1 min and 40 sec at 66° C, and 1 min at 72° C for extension. The reaction mixture was then mixed 1:1 with a solution con-

taining 20 mM EDTA, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol. Samples were heated at 95° C for 5 min, chilled on ice, and immediately loaded onto a 6% acrylamide/Tris-borate-EDTA gel containing 6% glycerol (volume/volume). Gels were run at 10 W (0.5 W/cm) for 3–4 hr at room temperature. Autoradiography was performed overnight at room temperature without intensifying screens. Genomic DNAs from control samples containing known wild-type and mutant p53 alleles were processed in parallel in every assay.

#### Chromosomal in situ hybridization

The methodology for interphase cytogenetic analysis has been previously described [12]. Briefly, sections from breast tumor tissue were dewaxed in xylene, dehydrated in graded alcohol, baked at 80° C for 1 h, and treated with 0.4% pepsin (Sigma, St. Louis, MO) in 0.2 N HCl for 30–55 min. Following denaturation at 94° C for 4 min, hybridization was carried out overnight in 60% formamide, 2X SSC containing 5% dextran sulphate, 1 mg/ml salmon sperm DNA, and 0.8–1.0 ng/ $\mu$ l probe. After hybridization, the slides were washed in 50% formamide in 2X SSC (pH 7.0) at room temperature and then washed in 0.1X SSC at 37° C. The hybridization signal was detected by the immunoperoxidase technique using the Vectastain ABC kit (Vector) and diaminobenzidine (DAB) as the chromogen substrate, as previously described [12]. Signals were quantitated as previously described [19]. The number of signal spots on a minimum of 100 nuclei in a given area was counted using previously described criteria [19]. A minimum of five randomly chosen areas were counted on each slide from each cell block. The CI was calculated by dividing the total number of signal spots by the number of nuclei counted. In brief, the chromosome index (CI) was calculated for defined histological regions by dividing the total number of signal spots by the total number of nuclei counted. For disomic cells, the CI for any given chromosome in our experience is 1.0. To account for minor technical differences in hybridization efficiency from one experiment to the next and from one region to another, a CI of  $\geq 1.20$

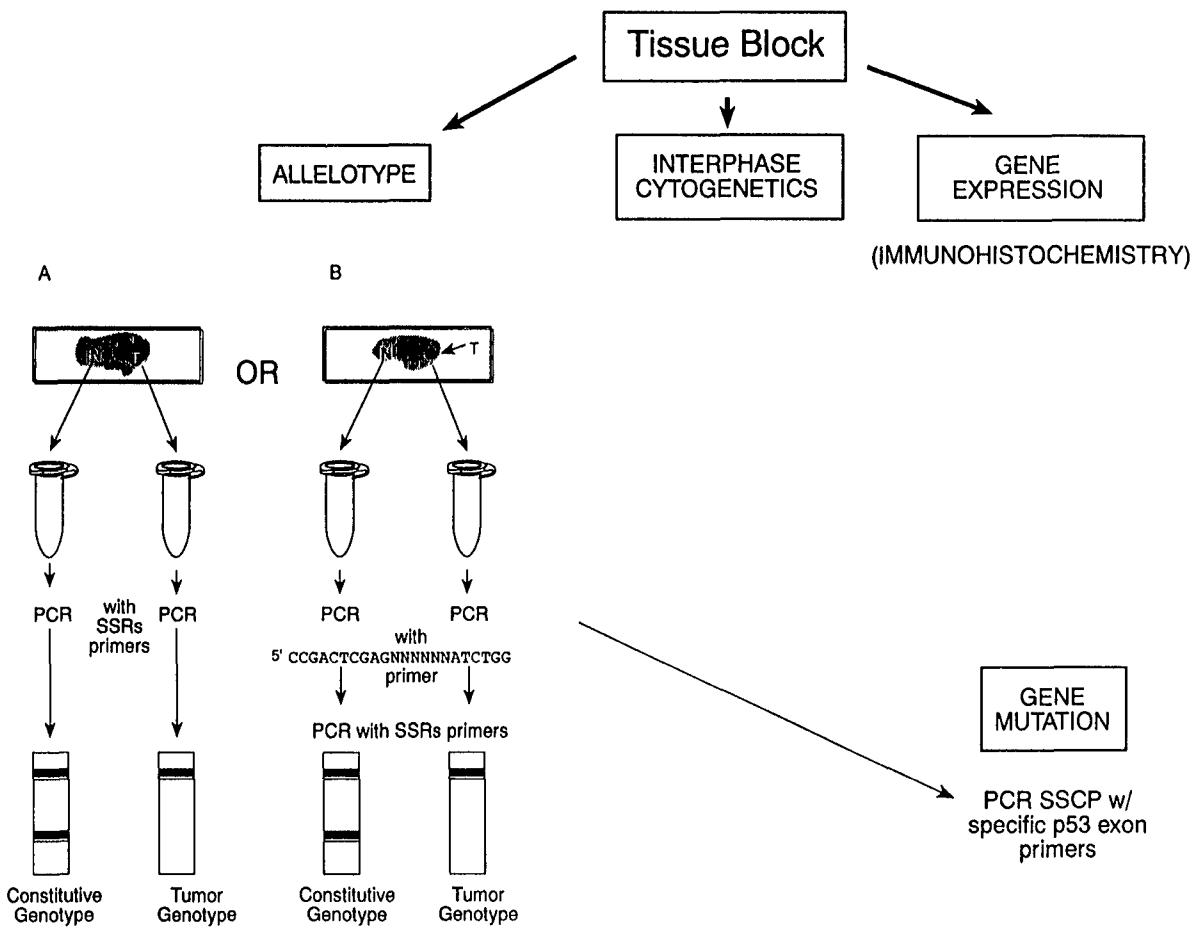


Fig. 1. Multiparameter analysis of breast cancer from paraffin sections to determine genotype phenotype correlations. A) DNA from normal (N) and tumor (T) areas of paraffin-embedded sections is obtained with the aid of microdissection. Normal DNA can also be obtained from a separate histological section of normal tissues from the same patient, e.g. unaffected lymph node. After microdissection of the area of interest the material is collected, purified and digested. PCR is performed using primers flanking specific microsatellite markers (SSRs). B) at right is shown the alternative protocol for extremely small samples using a few cycles of amplification with a universal primer mixture [17]. This DNA can also be used to screen for mutations such as in p53. Adjacent sections are used for interphase cytogenetics and immunohistochemistry.

was considered to represent polysomy and a CI of  $\leq 0.80$  monosomy.

When permitted by the size of the lesion, tumors were also analyzed by routine DNA flow cytometry to determine DNA index (DI).

#### Immunohistochemistry

p53 protein accumulation was analyzed by the avidin-biotin-peroxidase complex method using the D01 antibody (Oncogene Science), which detects

both mutant and wild-type p53 protein. Results were expressed as the approximate percentage of positive cells in random microscopic fields of observation. ERBB2 expression was detected with a commercially available antibody (Oncogene Science).

#### Results and discussion

We have developed a technique to analyze multiple chromosomal loci from single, microdissected, par-

affin-embedded sections. This involves PCR-mediated analysis of microsatellite length polymorphisms. Similar approaches were also recently developed by other laboratories [20]. We are now using the techniques described here to allelotype small preinvasive and invasive breast cancer lesions and then correlate the allelotypes with other tumor markers as well as cytogenetic changes such as numerical abnormalities of specific chromosomes (Fig. 1). To overcome the potential problem of normal DNA contribution from stromal or inflammatory cells, we microdissected areas of interest from each tissue section. From 5–8 µm tissue sections, we obtained enough template DNA to perform approximately 20–50 PCR reactions, which allowed the analysis of as many different chromosomal loci (Fig. 1A). The number of reactions depends on the size of the original sample, and certain primer sets require higher levels of template DNA. Thus, this general strategy was further improved by modifying a previously described DOP-PCR technique [17] to allow the analysis of even smaller samples and the gathering of larger amounts of template DNA. This facilitated the analysis of more chromosomal loci as well as the screening of specific gene mutations by PCR-SSCP. We obtained a minimum sample (1–2 µl) from the original template-DNA-containing solution by this alternative approach (Fig. 1B). This sample was subjected to a few cycles of PCR amplification with a universal degenerate primer, as described by Telenius *et al.* [17]. The resulting amplified products were used as template for amplification with the specific microsatellite flanking primers or the gene-specific primers (e.g. p53 amplimer panel). Figure 2 compares both of the methods described above. The allelic loss affecting marker D17S579 (upper allele) could be detected equally well by the direct technique (Fig. 2A) and the universal primer technique (Fig. 2B). This indicates that the proportionality of the alleles is preserved even after several cycles of whole genome amplification with a universal primer. This was further demonstrated by mixing at variable proportions two DNA samples, each homozygous for a different allele of marker D17S513, and comparing the sensitivity of both techniques, the direct approach and the universal primer method (Figs 2C and D,

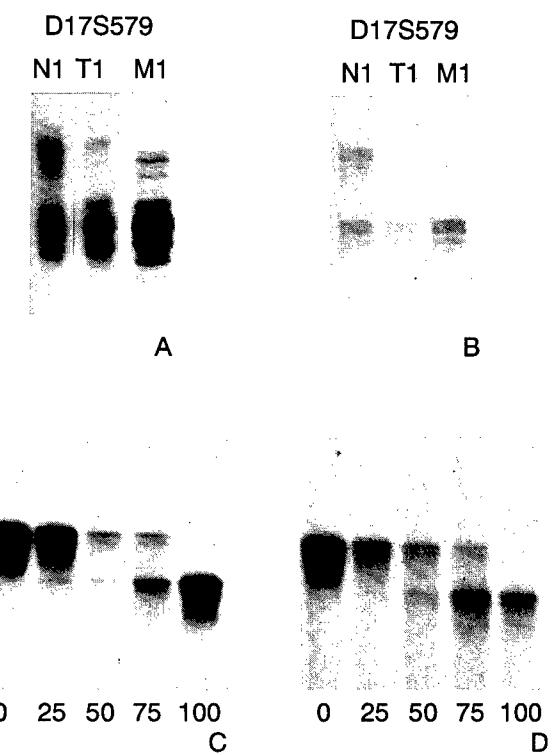


Fig. 2. Comparison of both techniques as shown in Fig. 1. A) Direct analysis of the D17S579 marker in patient 1, with loss of the upper allele in the tumor (T1) and in the corresponding metastasis (M1). At right B) is shown the analysis of the same D17S579 marker by the universal primer approach (Fig. 1B). As seen, the loss of the upper allele of this marker in T1 and M1 is preserved and detected using the universal primer approach.

Comparative analysis of mixtures of two alleles of D17S513 by using the direct method in C and the universal primer approach in D. Two DNA samples homozygous for D17S513 were mixed at various proportions prior to PCR, the percentage in the mixture of the sample with the smaller allele is shown at the bottom of each figure, see text for details.

respectively). The two samples with similar DNA concentrations were obtained from tissue sections were mixed at various proportions as indicated (Figs 2C, D) and were subjected to amplification using the direct approach (Fig. 2C). A 1 µl sample of the original mixtures was diluted in a 49 µl reaction volume for PCR amplification using the universal primer. Finally a 1 µl sample of this last reaction was used to amplify using the specific primer set for D17S513 (Fig. 2D). As can be observed both approaches yielded comparable results confirming that the universal primer method can detect alterations in the proportionality of the different alleles.

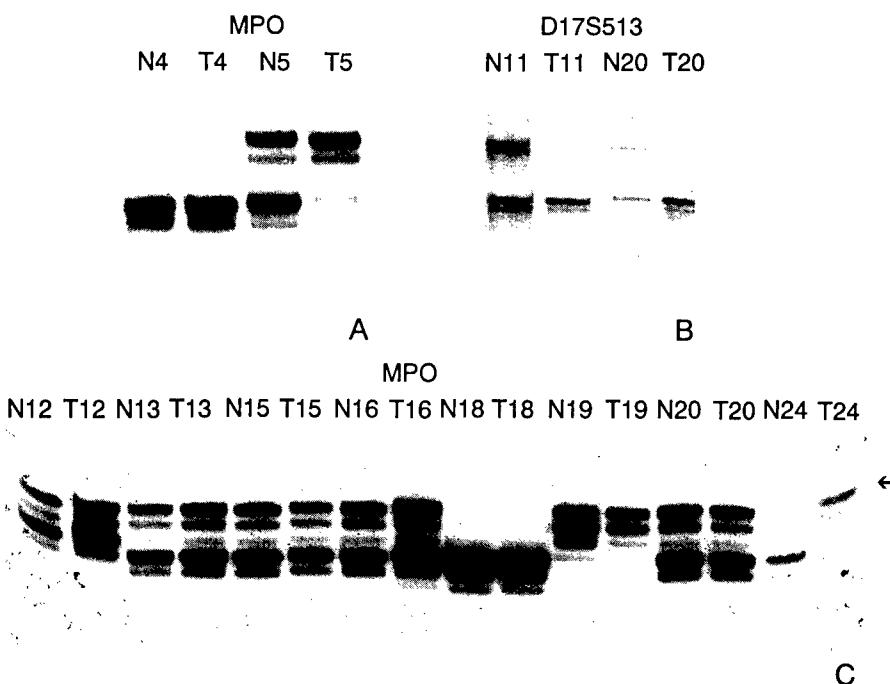


Fig. 3. Representative autoradiographs of multiple normal (N) and breast tumor (T) samples obtained from paraffin-embedded tissues demonstrating the analysis of chromosome 17 loci. In panels A and B note LOH in samples T5, T11, and T20. In panel C, note the generation of a novel allele (arrow) in the tumor T24; we observed the same phenomenon with other markers in this patient (microsatellite instability). PCR-amplified microsatellites with one primer end labeled were separated on a 7% polyacrylamide sequencing gel.

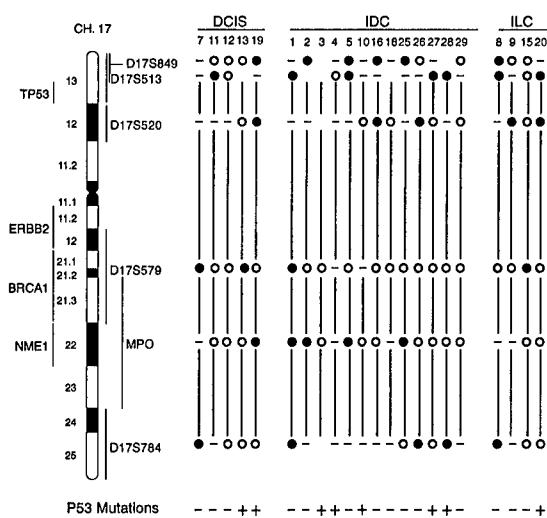


Fig. 4. Schematic representative summary of chromosome 17 allelotype and p53 mutation analysis of various breast cancer samples previously analyzed by means of microsatellite polymorphism in paraffin sections. Open circles, no LOH; closed circles, LOH or allelic imbalance; -, noninformative. p53 mutations were detected by PCR-SSCP analysis.

Figure 3 shows a representative analysis of multiple loci from the q and p arms of human chromosome 17 in various invasive and *in situ* breast carcinomas by the direct technique. Figure 4 shows the results of our detailed chromosome 17 analysis of 22 breast tumors (5 ductal carcinomas *in situ*, 13 invasive ductal carcinomas, and 4 invasive lobular carcinomas).

We also evaluated the sensitivity of the universal primer technique in detecting specific gene mutations. In this case we analyzed the same 22 tumors for p53 gene mutations by PCR-SSCP assay. We used 5 ng samples of template DNA from normal human DNA as negative controls and 5 ng samples from cell lines with known mutations in each of the p53 exons as positive control (e.g. Colo-320 for exon 7, BT-474 for exon 8). The control and tumor samples were first subjected to universal primer amplification as described in Methods. A 1–2  $\mu$ l sample of the resulting products was then subjected to a second PCR using the end-labeled p53 exon-specific primers. After a final denaturation, the

PCR products were separated on a 6% polyacrylamide gel under nondenaturing conditions. Representative results are shown in Fig. 5. Of the 22 tumors analyzed for mutations in p53 exons 5–8, we detected mobility shifts in 8 (36%) samples: 2 ductal carcinomas *in situ*, 4 invasive ductal carcinomas, and 1 lobular carcinoma (Fig. 4 lower panel).

Adjacent sections from 14 of the tissue blocks were also analyzed for chromosome 17 copy number by interphase cytogenetics CISH and expression of p53 and ERBB2 by immunohistochemistry. The results of these studies are shown in Table 1. Based on the data shown in Fig. 4 and Table 1, we concluded that allelic losses and imbalance already were occurring at the carcinoma *in situ* stage, as previously reported [21]. In several cases we observed allelic losses or imbalance affecting the 17p13 region, close to the p53 locus, but in several other tumors this event and p53 mutations were dissociated as observed by other authors [22]. However, tumors showing significant levels of p53 protein accumulation such as tumors 7, 10, and 19 also showed mobility shifts in the p53 PCR-SSCP assay. We also observed only a modest number of losses involving the 17q21-22 region, lower than previously reported by others [5].

The results also show that interphase cytogenetic analysis supplements the information gained from ploidy analysis by DNA flow cytometry alone. In many cases where flow cytometry could not be performed because the lesions were too small for gross observation, CISH techniques allowed the copy numbers of specific chromosomes to be estimated (e.g. tumors 5, 10, 16, 19, and 24). Furthermore, difference in copy number of chromosomes between adjacent but phenotypically distinct regions could be determined: e.g. tumor 10, the *in situ* component was polysomic (CI 1.66) for chromosome 17, but the invasive component was disomic (CI 1.10).

In summary, we have developed and applied a methodology for analyzing large numbers of chromosomal loci from single paraffin-embedded sections of small preinvasive and invasive breast cancer lesions. The basic technique involves tissue microdissection and microsatellite length polymorphism analysis. We have further improved this approach conducting a first round of DNA PCR

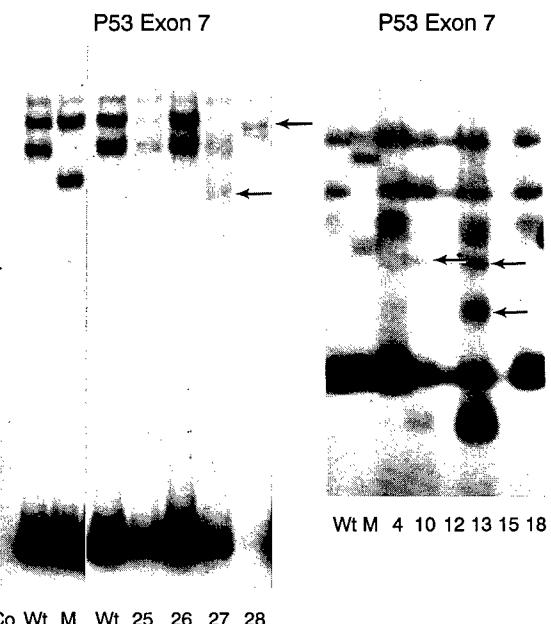


Fig. 5. Representative PCR-SSCP analysis of p53 exons 6 and 7 from a DNA template obtained from paraffin-embedded breast cancer tissues using the universal primer method. WT; wild type, M; positive control DNA for mutation in the corresponding exon. Note the clear shift in band mobility in some of the positive samples as indicated by arrows.

amplification with a degenerate universal primer for total genome amplification and then with specific microsatellite primers. We observed that the DNA so obtained preserved the proportionality of the different alleles as found in the original sample. We also determined that DNA obtained from the same lesions and amplified with the universal primer could be used to screen for specific gene mutations such as in p53. In addition, tissue sections adjacent to those used for the micromolecular analysis were successfully used to assess specific chromosome copy number by interphase cytogenetic analysis (CISH) and to analyze the expression of specific genes by immunohistochemistry.

This type of comprehensive approach using archival paraffin-embedded tissues will allow correlation of genetic changes (at both the chromosomal and molecular levels) with their phenotypic consequences in the same preinvasive and invasive lesions. Such an approach will also allow us to dissect the specific events involved in the multistep process of breast carcinogenesis.

Table 1. Analysis of multiple genotype and phenotype characteristics from breast cancer paraffin sections

Sample	Pathology <sup>a</sup>	Nuclear grade <sup>c</sup>	LOH or imbalance <sup>d</sup>		p53 mutation <sup>e</sup>	p53 accumulation <sup>f</sup>	c-erb B-2 expression <sup>g</sup>	DNA index <sup>h</sup>	Chromosome 17 CI <sup>i</sup>
			17p	17q					
4	IDC	I	○	ni	+(E6)	nd	nd	1.99	0.86
5	IDC	I	(2)●	(1)●	-	-	-	nd	0.91
10	IDC/DCIS	II	○	○	+(E7)	5%	-	nd	IDC 1.10; DCIS 1.66
16	IDC	I	(2)●	○	-	5%	-	nd	1.60
7	DCIS <sup>b</sup>	II	ni	(2)●	-	-	-	1.0	1.09
11	DCIS	I	(1)●	○	-	-	+	2.22	2.24
12	DCIS	I	○	○	-	-	+	1.36	nd
13	DCIS	I	○	(1)●	+(E7)	50%	+	1.13	1.19
19	DCIS	II	(1)●	○	+(E8)	20%	-	nd	1.93
24	DCIS	II	MI <sup>j</sup>	MI <sup>j</sup>	nd	-	-	nd	nd
8	ILC	III	(2)●	(1)●	-	1%	-	1.16	1.12
9	ILC	II	(1)●	○	-	1%	-	1.0	1.05
15	ILC	II	○	(1)●	-	-	-	0.93	1.02
20	ILC/LCIS	II	(2)●	○	+(E8)	nd	-	1.65	1.21

<sup>a</sup> Abbreviations: IDC, invasive ductal carcinoma; DCIS, ductal carcinoma *in situ*; ILC, invasive lobular carcinoma; LCIS, lobular carcinoma *in situ*.

<sup>b</sup> All DCIS lesions were comedo DCIS with the exception of Sample # 7 which is a non-comedo lesion.

<sup>c</sup> According to Black's nuclear grading system in which I = poorly differentiated, II = moderately differentiated and III = well differentiated.

<sup>d</sup> According to microsatellite analysis. ●: loss or allelic imbalance, in parenthesis number of markers affected per arm; ○, no loss; ni, non informative.

<sup>e</sup> Exon affected, as determined by PCR-SSCP; shown in parentheses.

<sup>f</sup> Determined by immunohistochemistry using an antibody that recognizes both wild-type and mutant p53 protein. -, negative for accumulation; +, accumulation expressed as approximate percentage of positive cells; nd, not determined.

<sup>g</sup> Determined by immunohistochemistry.

<sup>h</sup> Determined by DNA flow cytometry from fresh specimens.

<sup>i</sup> Determined by chromosomal *in situ* hybridization with chromosome 17 centromeric probe.

<sup>j</sup> MI, microsatellite instability.

## Acknowledgements

Supported by U.S. Army Award DAMD 17-94J-4078 and NIH grant P20 CA58186. We thank John Riley and Judy Ing for artwork and Michelle Gardiner for secretarial assistance.

## References

- Callahan R, Campbell G: Mutations in human breast cancer: an overview. *J Natl Cancer Inst* 81: 1780-1786, 1989
- McGuire WL, Naylor SL: Loss of heterozygosity in breast cancer: cause or effect? *J Natl Cancer Inst* 81: 1764-1765, 1989
- Chen-L-C, Kurisu W, Ljung BM, Goldman ES, Moore II D, Smith HS: Heterogeneity for allelic loss in human breast cancer. *J Natl Cancer Inst* 84: 506-510, 1992
- Weber JL, May PE: Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am J Hum Genet* 44: 388-396, 1989
- Futreal PA, Söderkvist P, Marks JR, Iglehart JD, Cochran C, Barrett JC, Wiseman RW: Detection of frequent allelic loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite length polymorphisms. *Cancer Res* 52: 2624-2627, 1992
- Jones MH, Nakamura Y: Deletion mapping of chromosome 3p in female genital tract malignancies using microsatellite polymorphism. *Oncogene* 7: 1631-1634, 1992

7. Aaltonen LA, Peltomäki P, Leach FS, Sjöström P, Pylkkänen L, Mecklin J-P, Järvinen H, Powell SM, Jen J, Hamilton SR, Petersen GM, Kinzler KW, Vogelstein B, de la Chappelle A: Clues to the pathogenesis of familial colorectal cancer. *Science* 260: 812–816, 1993
8. Hopman AHN, Moesker O, Smeets AWGB, Pauwels RP, Vooijs GP, Ramaekers FC: Numerical chromosome 1, 7, 9 and 11 aberrations in bladder cancer detected by *in situ* hybridization. *Cancer Res* 51: 644–651, 1991
9. Devilee P, Thierry RF, Kievits T, Kolluri R, Hopman AHN, Willard HF, Pearson PL, Cornelisse CJ: Detection of chromosome aneuploidy in interphase nucleic from human primary breast tumors using chromosome-specific repetitive DNA probes. *Cancer Res* 58: 5825–5830, 1988
10. Emmerich P, Jauch A, Hofmann M-C, Cremer T, Walt H: Interphase cytogenetics in paraffin embedded sections from human testicular germ cell tumor xenografts and in corresponding cultured cells. *Laboratory Investigation* 61: 235–242, 1989
11. Walt H, Emmerich P, Cremer T, Hofmann M-C, Bannwart F: Supernumerary chromosome 1 in interphase nuclei of atypical germ cells in paraffin-embedded human seminiferous tubules. *Laboratory Investigation* 61: 527–531, 1989
12. Dhingra K, Sahin A, Supak J, Kin SY, Hortobagyi G, Hittelman WN: Chromosome *in situ* hybridization on formalin-fixed mammary tissue using non-isotopic, non-fluorescent probes: technical considerations and biological implications. *Breast Cancer Res and Treatment* 23: 201–210, 1992
13. Oliphant AR, Wright EC, Swensen J, Gruis NA, Goldgar D, Skolnick MH: Dinucleotide repeat polymorphism at the D17S513 locus. *Nucleic Acids Res* 19: 4794, 1991
14. Hall JM, Friedman L, Guenther C, Lee MK, Weber JL, Black DM, King MC: Closing in on a breast cancer gene on chromosome 17q. *Am J Hum Genet* 50: 1235–1242, 1992
15. Polymeropoulos MH, Xiao H, Rath DS, Merril CR: Dinucleotide repeat polymorphism at the human gene of the light and heavy chains of myeloperoxidase glycoprotein (MPO). *Nucleic Acids Res* 19: 1961, 1991
16. Weissenbach J, Gyapay G, Dib C, Vignal A, Morissette J, Millasseau P, Vaysseix G, Lathrop M: A second-generation linkage map of the human genome. *Nature* 359: 794–801, 1992
17. Telenius H, Carter NP, Bebb CE, Nordenskjold M, Ponder BA, Tunnacliffe A: Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. *Genomics* 13: 718–725, 1992
18. Orita M, Iwahana H, Kanazawa H, Hayashi K, Seklyre T: Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphism. *Proc Natl Acad Sci USA* 86: 2766–2770, 1989
19. Dhingra K, Sneige N, Pandita TK, Johnston DA, Lee JS, Emami K, Hortobagyi GN, Hittelman WN: Quantitative analysis of chromosome *in situ* hybridization signal in paraffin-embedded tissue sections. *Cytometry* 16: 100–112, 1994
20. O'Connell P, Pekkel V, Fuqua S, Osborne CK, Allred DC: Molecular genetic studies of early breast cancer evolution. *Breast Cancer Research Treatment* 32: 5–12, 1994
21. Radford DM, Fair K, Thompson AM, Ritter JH, Holt M, Steinbrueck T, Wallace M, Wells SA, Donis-Keller HR: Allelic loss on chromosome 17 in ductal carcinoma *in situ* of the breast. *Cancer Res* 53: 2947–2950, 1993
22. Deng G, Chen L-C, Schott DR, Thor A, Bhargava V, Ljung B-M, Chew K, Smith HS: Loss of heterozygosity and *p53* gene mutations in breast cancer. *Cancer Research* 54: 499–505, 1994

## Deletion Map of Chromosome 16q in Ductal Carcinoma *in Situ* of the Breast: Refining a Putative Tumor Suppressor Gene Region<sup>1</sup>

Taiping Chen, Aysegul Sahin, and C. Marcelo Aldaz<sup>2</sup>

Department of Carcinogenesis, The University of Texas M. D. Anderson Cancer Center, Science Park-Research Division, Smithville, Texas 78957 [T. C., C. M. A.], and Department of Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [A. S.]

### Abstract

Allelic losses or imbalances affecting chromosome arm 16q appear to be early genomic abnormalities in breast carcinogenesis, because they were observed in a significant number of breast ductal carcinoma *in situ* lesions in our previous study (Aldaz *et al.*, Cancer Res., 55: 3976-3981, 1995). To define the minimum region of loss of heterozygosity (LOH), we generated a high-resolution allelotype of 35 ductal carcinoma *in situ* cases and completed a deletion map of chromosome 16q by means of paraffin-embedded tissue microdissection and PCR microsatellite analysis of 22 markers. We observed a strikingly high frequency of LOH in 16q, with 31 of 35 tumors (89%) affected. We identified three distinctive areas with high LOH. Two areas were described previously and correspond to 16q21 and 16q24.2-qter. The third and most commonly affected area spanned the region from marker D16S515 to marker D16S504. The most affected locus was at D16S518, in which LOH was observed in 20 of 26 informative cases (77%), and we estimate that it lies in subregion q23.3-q24.1. The region of highest LOH spanned approximately 2-3 Mb, as determined by a yeast artificial chromosome contig reported to cover this region. Such a high frequency of LOH at a preinvasive stage of breast cancer suggests that a candidate tumor suppressor gene or genes at this location may play an important role in breast carcinogenesis.

### Introduction

Breast cancer is the most common cancer among women, accounting for approximately 46,000 deaths in the United States each year (1). Numerous studies have focused on the identification and analysis of specific gene mutations and chromosome abnormalities in sporadic breast cancer, but to date no clear model of the critical events or delineation of primary abnormalities has emerged (2). DCIS<sup>3</sup> of the breast is known to be a preinvasive stage of breast cancer and is probably the precursor of infiltrating breast cancer (3). Thus, genetic alterations shown at this stage might indicate association with early events in malignancy or invasiveness.

LOH at specific chromosomal loci has been considered part of the indirect evidence for postulating the existence of possible tumor suppressor genes within those specific chromosome regions. It is known that several mechanisms, such as chromosomal deletions, monosomy, mitotic recombination, and unbalanced translocation, can lead to the loss of alleles in tumors (4). Hypothetically, the remaining allele of the tumor suppressor gene in question could be rendered inactive by events occurring at the gene level, such as specific point mutations or other types of inactivating mutations. Usually, LOH at

specific chromosome regions affects not only the putative tumor suppressor gene but also neighboring genes or genetic markers that are used as indicators to track down the minimum area of LOH.

In a previous report, we described the development of a technical approach that allowed us to generate LOH analyses (allelotypes) from paraffin-embedded tissue samples (5). The basic technique involves tissue microdissection and microsatellite length polymorphism analysis. Using this micromolecular approach, we compared the allelotypes of *in situ* and invasive breast cancer lesions (6). We observed that specific chromosome arms are more frequently affected by allelic losses and imbalances at preinvasive stages of breast cancer. In particular, allelic losses affecting chromosome 16q, as well as chromosomes 17p, 17q, and 7, appear to be early genomic abnormalities because they were observed in a significant number of DCIS lesions (6).

LOH on chromosome 16q at various frequencies has been widely reported in breast (7-10), prostate (11), hepatoblastoma (12), and Wilms' tumors (13). In particular, two regions on chromosome 16q have been revealed to have a very high frequency of LOH in breast cancer; one maps to region 16q22.1 and the other to 16q24.2-qter (8-10).

In the study reported here, we extended our previous observations (6) using PCR microsatellite-length polymorphism analysis and precise tissue microdissection of paraffin-embedded tumor samples to generate a high-resolution deletion map of chromosome 16q in breast DCIS. We were able to identify a region of approximately 2-3 Mb as the location of a putative tumor suppressor gene of possible relevance in the development of breast cancer.

### Materials and Methods

**Tumor Samples.** DCIS samples were obtained from paraffin-embedded tissue blocks from the archives of the Department of Pathology of The University of Texas M. D. Anderson Cancer Center. A total of 35 cases of tumors collected and diagnosed as pure breast DCIS by our collaborating pathologist (A. S.) was analyzed. DCIS samples were classified by nuclear grade and by the presence (comedo) or absence of necrosis. Twelve tumors were classified as poorly differentiated with high nuclear grade; the remaining 23 samples were classified as moderate and well differentiated with lower nuclear grades (moderate and low nuclear grades). Twenty-one of the samples showed evidence of necrosis; the remaining 14 showed no necrosis. We did not include any DCIS with infiltrating components.

**Paraffin-embedded Tissue Microdissection.** The basic technical approach has been described previously (5). Minor modifications were introduced to improve the efficiency of microdissection. Briefly, one to three 5-8- $\mu$ m-thick paraffin sections were stained and microdissected. Using companion H&E-stained slides as a reference, tumor cells were microdissected using a fine-point surgical blade (No. 11) under an inverted microscope. The edges of tumor area and stroma were cleared of debris using the same blade and blown with a stream of compressed air. A new blade was then used to dissect normal tissue the same way.

**DNA Preparation.** Samples were rehydrated, and DNA was extracted by incubating in 200- $\mu$ l Instagene chelex matrix solution (Bio-Rad) containing 60

Received 9/9/96; accepted 10/29/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported by United States Army Breast Cancer Program Grant DAMD 17-94-J-4078.

<sup>2</sup> To whom requests for reprints should be addressed, at Department of Carcinogenesis, The University of Texas M. D. Anderson Cancer Center, Science Park-Research Division, P. O. Box 389, Smithville, Texas 78957. Phone: (512) 237-2403; Fax: (512) 237-2475.

<sup>3</sup> The abbreviations used are: DCIS, ductal carcinoma *in situ*; LOH, loss of heterozygosity; YAC, yeast artificial chromosome.

$\mu$ g of proteinase K in a shaking incubator at 37°C overnight. After proteinase K digestion, samples were boiled for 10 min, vortexed, and centrifuged at >7000  $\times$  g for 5 min. Aliquots of the supernatant (5  $\mu$ l each) were used for PCR amplification.

**PCR Microsatellite Analysis.** The primers for highly polymorphic human microsatellite repeats listed in Table 1 were purchased from Research Genetics (Huntsville, AL). Before PCR reactions, the forward primer was end labeled using T4 polynucleotide kinase (Promega) and [ $\gamma$ -<sup>32</sup>P]ATP (DuPont New England Nuclear). Each PCR reaction was performed in a 20- $\mu$ l reaction volume containing 150  $\mu$ M each dNTP, 1 unit of Taq polymerase (Promega), 1 $\times$  Taq buffer (Promega), 1.5 mM MgCl<sub>2</sub>, 1 pmol-labeled primer, and 2.5 pmol of unlabeled forward and reverse primers. A hot-start procedure was used in which template and primers were denatured at 96°C for 5 min. Afterward, the remaining reaction constituents were added for 35–40 cycles at 94°C for 40 s, 55°C for 30 s, and 72°C for 30 s, and a final elongation step at 72°C for 5 min. Products were electrophoresed on 7% polyacrylamide sequencing gels at 90 W of constant power for 2–3 h. Gels were dried at 65–70°C for 1–2 h and exposed to X-ray film from 4 h to overnight. As necessary, for certain primer sets, the amplification conditions were further optimized by adjusting the MgCl<sub>2</sub> concentration in the reaction buffer.

The sample was considered to have partial LOH, or allelic imbalance, if the normal signal intensity of one allele was diminished by approximately half or more in relation to the remaining allele. Complete LOH was defined as a decrease of 90% or more in the signal intensity of one allele relative to the other. YAC clones spanning the region of interest were purchased from Research Genetics.

## Results

**Microdissection of Paraffin-embedded Ductal Carcinoma *in Situ*.** To optimize the microdissection of tumor and normal samples, the sections were stained before dissection. In this way, exact areas of tumor and normal tissue could be dissected from the same slide. Fig. 1 shows three foci of DCIS (case 59) before (A) and after (B) microdissection. For each slide, separate blades were used to dissect tumor and normal samples, and a stream of compressed air was used to clear away debris before dissecting the normal tissue. Using this approach, we generated from a single slide relatively pure tumor and normal genomic DNA pools that could serve as templates for approximately 20–30 PCR reactions.

**Allelic Loss and Deletion Map on Chromosome 16q.** We analyzed a total of 35 breast DCIS cases for LOH using a panel of 22

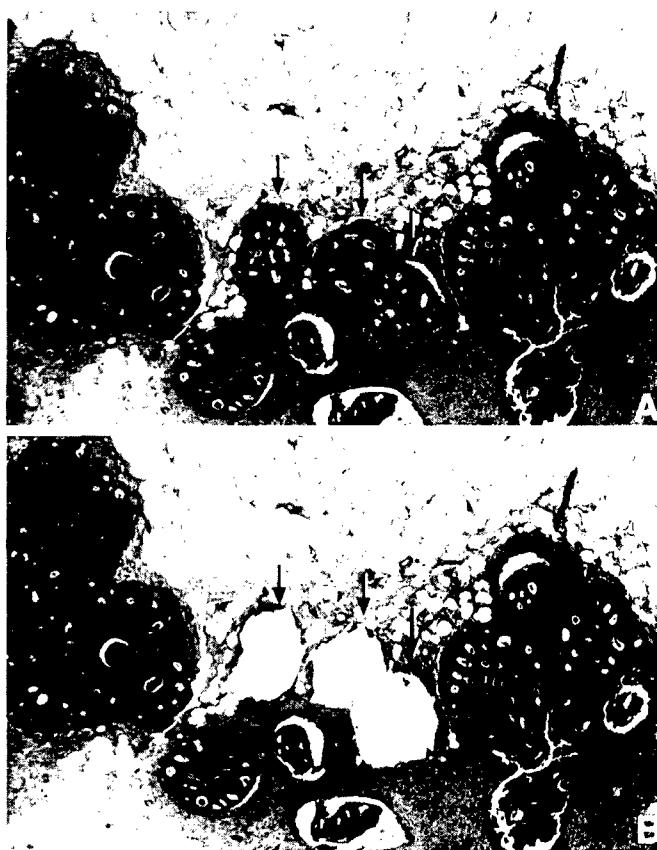


Fig. 1. Representative microdissection of a DCIS sample from paraffin-embedded tissue section. A, before microdissection; note the three central structures (arrows). B, same area after microdissection (arrows).

microsatellite markers as summarized in Table 1 and Fig. 2. Thirty-one of these samples (89%) showed LOH, or allelic imbalance, in at least one or more chromosome 16 loci. Fig. 2 schematically displays the chromosome 16 allelotype of the individual DCIS tumors. We observed no correlation between the occurrence or pattern of LOH and the histopathological classification. (i.e., DCIS samples with or

Table 1 Chromosome 16 LOH in ductal carcinoma *in situ* of the breast

Locus	Cumulative linkage map distance (cM) <sup>a</sup>	Cytogenetic location <sup>b</sup>	No. of cases	Tumors with LOH/Informative cases	% LOH
D16S407		p13.13	34	2/33	6
D16S420	47	p12.3	31	3/24	13
D16S285		q12.1	34	4/24	17
D16S261		q12.1	34	6/20	30
D16S390		q12.2	32	5/23	22
D16S533		q21	33	7/23	30
D16S400	89	q21	32	7/11	64
D16S503	88	q21	28	10/21	48
D16S398		q22.1	34	13/28	46
D16S421	92	q22.1	32	12/22	55
D16S512	98	q22.1	33	9/21	43
D16S260		q22.2	29	8/16	50
D16S395		q23.1–q23.2	35	14/26	54
D16S515	100	q22.3–q23.1	33	13/23	57
D16S518	103	q23.1–q24.2	33	20/26	77
D16S516	108	q24.1	30	14/21	67
D16S504	109	q24.1	32	15/20	75
D16S507	113	q23.2	35	7/19	37
D16S393		q24.1	32	10/23	43
D16S422	119	q24.2	29	10/21	48
D16S402	120	q24.2	32	14/19	74
D16S413	137	q24.3	35	10/27	37

<sup>a</sup> According to Genethon Linkage Map (March 1996).

<sup>b</sup> According to GDB (August 1996).

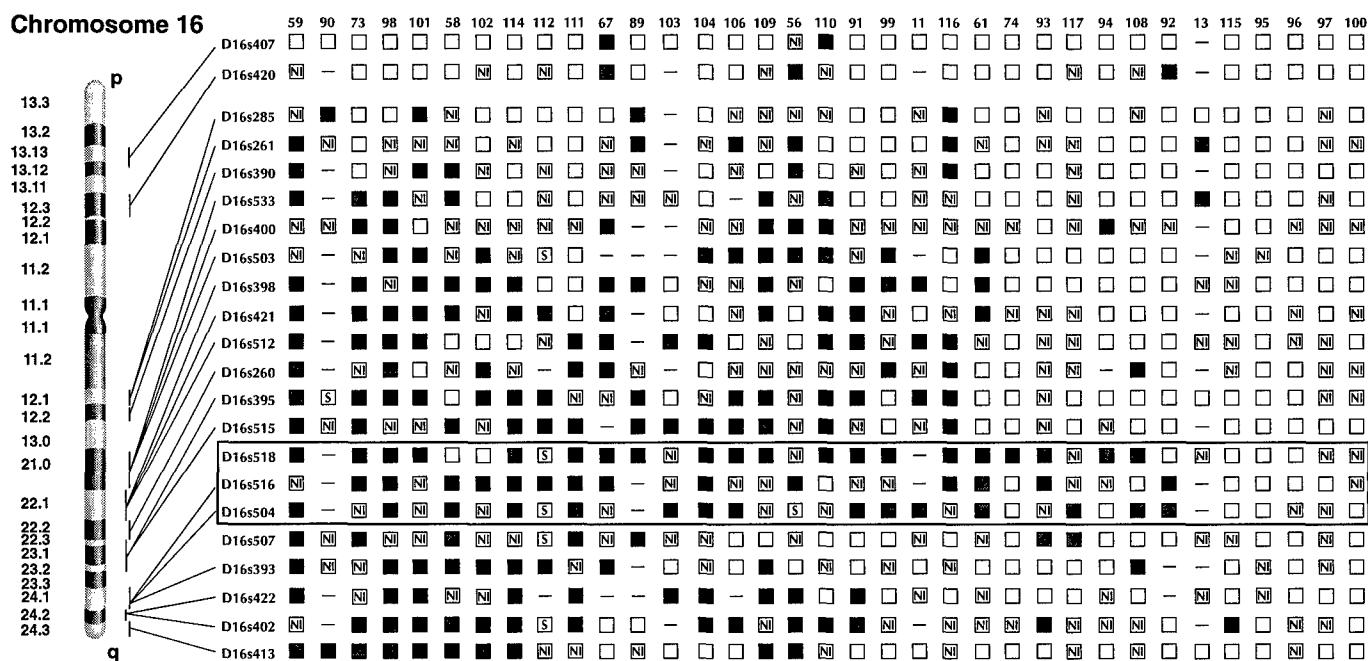


Fig. 2. Schematic representation of chromosome 16q allelotype in breast DCIS. Numbers at the top of the figure represent each individual tumor. ■, LOH; □, allelic imbalance; □, no LOH; NI, noninformative locus; S, shift in allele size.

without necrosis and with high or low nuclear grades showed LOH at multiple loci in chromosome 16q.) As can be observed, approximately one-third of the cases displayed large terminal deletions or recombinations involving most or all of the whole chromosome 16q arm (Fig. 2). The remaining two-thirds of LOH appeared to be the result of mostly interstitial deletions or more complex recombination events. By overlapping the LOH pattern of the various tumors, we could determine that the region between markers D16S515 and D16S504 (box in Fig. 2) was the most commonly affected area. Fig. 3 shows representative autoradiographs demonstrating allelic losses in tumor-derived DNA between 16q21 and 16q24. Clear patterns of allelic losses, as shown, were observed throughout the experiment.

Table 1 summarizes the information gathered on the 22 loci studied. Loci are displayed in linear order according to the integrated chromosome 16 physical and genetic map (14). Three distinct regions were observed to have a very high percentage (~70% or above) of allelic losses among informative DCIS samples. The main region extended approximately from 16q23.1 to 16q24.1, which spans the area including markers D16S515 (57% frequency of LOH), D16S518 with losses in 20 of 26 informative DCIS samples (77%), D16S516 (67%), and D16S504 (75%). The two other areas of high LOH were 16q24.2, including the D16S402 locus with a 74% LOH (14 of 19 informative cases), and 16q21, including D16S400 (64% LOH; 7 of 11 informative cases). Other loci in the p arm and in the q arm more proximal to the centromere showed a much lower frequency of involvement.

As indicated, D16S518 was the most commonly affected locus in the chromosome 16q arm. To estimate the approximate physical size of the region with the highest frequency of overlapping deletion, the region of interest was compared with a partial YAC contig that reportedly spans this area (Fig. 4; Ref. 14). We confirmed the location of the microsatellite markers D16S518, D16S516, and D16S504 to YAC clones 933h2 and 972d3 as shown. This area appears to span approximately 2–3 Mb, and is very likely located within chromosome bands 16q23.3–q24.1.

## Discussion

DCIS was always suspected to be the most probable precursor to invasive carcinoma (3). Page and Dupont (3) found a greatly increased risk of subsequent invasive breast cancer in women with a history of DCIS positive biopsy. Recently, several laboratories have provided molecular evidence that further substantiates the model of progression from DCIS to invasive breast cancer (15–17). Nevertheless, we still know very little about the role of specific genetic abnormalities at preinvasive stages of breast cancer development. We have recently demonstrated that specific chromosome arms are more frequently affected by allelic losses at the DCIS stage (6). On the basis of these results, we postulated that these genetic alterations in DCIS are early events and may play an important role in the genesis of invasive breast cancer. In that study, we observed that marker D16S413 located on telomeric band 16q24.3 was significantly affected by allelic losses when compared with other loci. This prompted us to extend our analysis on the q arm of chromosome 16 by generating a high-resolution allelotype of this chromosome arm.

Chromosome 16q has been suggested as a site for the occurrence of primary cytogenetic structural abnormalities in the development of breast cancer (18, 19). In particular, 16q was shown to participate systematically in nonrandom translocations with chromosome 1 and to have frequent deletions (2). Furthermore, breast cancer allelotypic studies have systematically shown the common occurrence of allelic losses affecting the chromosome 16q arm. In addition to our observations (6), other investigators have also reported the occurrence of frequent allelic losses affecting chromosome 16q in DCIS (9, 20).

It has been suggested that more than one putative tumor suppressor locus of interest in breast cancer may reside in 16q. At least two regions of chromosome 16q have been reported previously to have consistent LOH: 16q21 and 16q24.2–qter (2, 8–10). Here, we report that 31 of 35 DCIS tumors (89%) showed allelic losses in one or more loci of the long arm of chromosome 16. This is a very high incidence when compared with approximately 50% or lower frequency of LOH seen in other studies of invasive breast carcinomas (8–10). We can

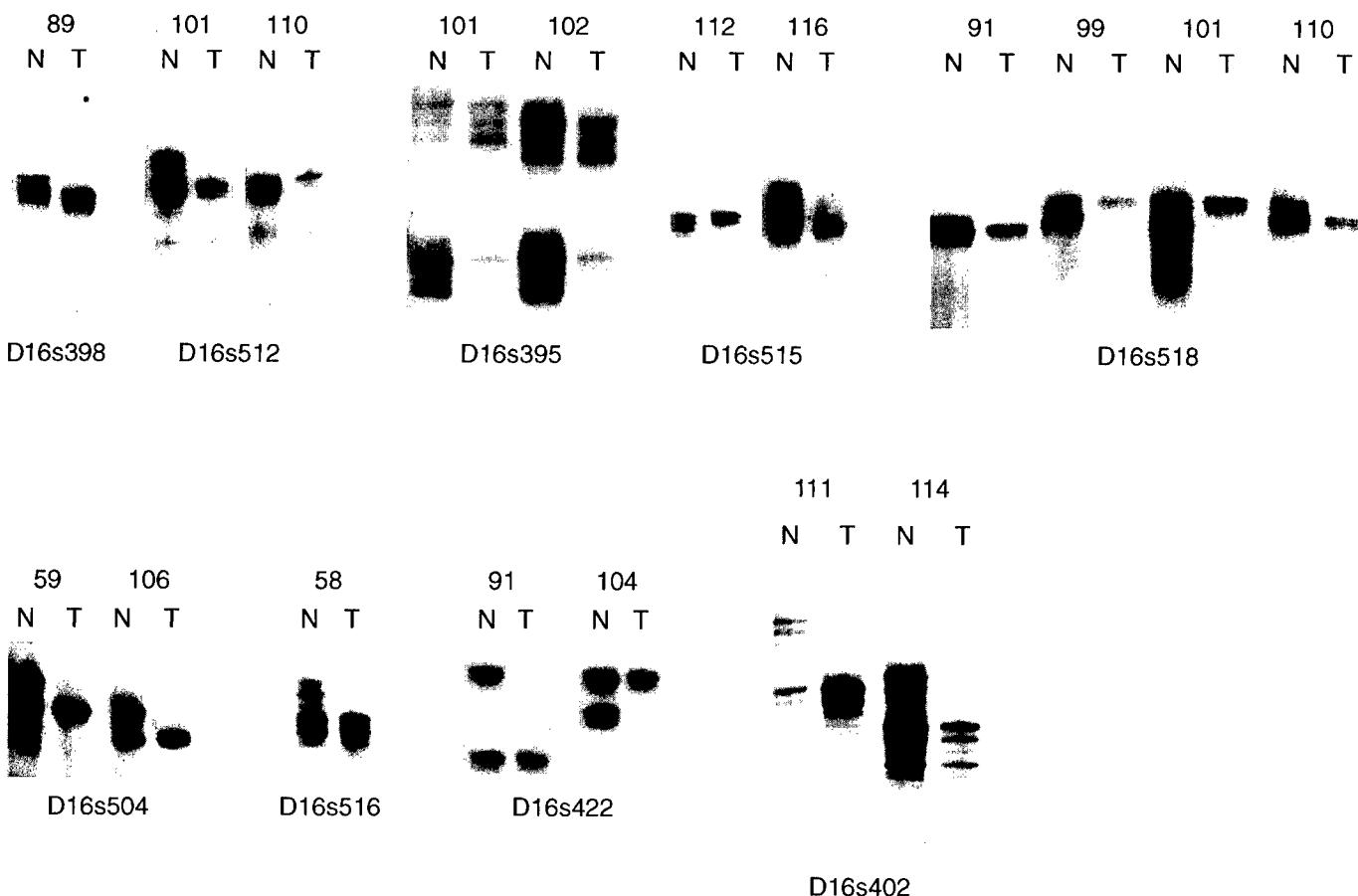


Fig. 3. Representative autoradiographs demonstrating LOH and allelic imbalance at various chromosome 16q loci as indicated. *N*, normal tissue; *T*, tumor (DCIS) tissue.

explain this difference by our use of very precise tissue microdissection in our study, which improved the separation of tumor DNA from normal stromal contaminants. This is particularly important when dealing with small islands of tumor cells such as in DCIS. In addition, in our study we used highly polymorphic microsatellite markers that provide a high number of informative samples.

In our analysis of DCIS lesions, we identified three distinct regions with a very high percentage (~70% or above) of allelic losses among informative DCIS samples. Two of them agree with previously described areas: 16q21 at locus *D16S400* and 16q24.2 at locus *D16S402*. However, the region with the highest incidence of LOH observed in our study spanned the region between markers *D16S515* and *D16S504* (see Table 1). Within this region, the *D16S518* locus was the most frequently affected, with 20 of 26 DCIS tumors (77%) at this site showing LOH.

These observations strongly suggest that a putative tumor suppressor gene or genes may be harbored at or near this locus. Furthermore, the incidence of allelic loss at *D16S518* could potentially be higher, because a few tumors that preserved heterozygosity at this locus also showed losses of flanking markers (e.g., tumors 58 and 102). This could be due to homozygous deletions affecting this region, changes that are very difficult to judge because of the nature of the PCR-mediated approach we used.

On the basis of a partial YAC contig reported to span the region of interest (14), we were able to estimate that the minimum region with the highest frequency of LOH is no larger than 2–3 Mb (Fig. 4). Furthermore, based on the cytogenetic location of markers *D16S516* and *D16S504* and the distance to *D16S518*, this area should be contained within bands 16q23.3–q24.1. This region appears different from another more distal area of frequent LOH at locus *D16S402* in band 16q24.2. This observation is substantiated by the fact that both areas are 17 cM apart based on the Genethon Linkage Map (March 1996) and several megabases away based on the comprehensive chromosome 16 physical and genetic map (14).

It will be particularly important now to analyze other, less advanced hyperplastic breast lesions for the occurrence of allelic losses in the chromosome regions identified in this report. This analysis will be useful in improving our understanding of breast carcinogenesis and may help in the identification of markers with diagnostic-prognostic significance.

In summary, this study has shown that chromosome 16q is highly affected by allelic losses in breast DCIS. We refined the location of the minimal deleted region that we found most commonly affected at 16q23.3–q24.1, locus *D16S518*, a locus that to our knowledge was not reported previously. These data indicate the existence of a putative

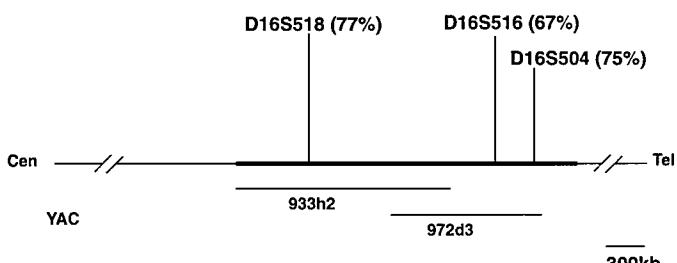


Fig. 4. Minimum area of highest LOH and partial YAC contig spanning the region as reported by Hudson *et al.* (14). Numbers in parentheses indicate percentage of LOH at the indicated marker.

tumor suppressor gene or genes, most probably located within a 2-3-Mb region. Two other regions of interest for LOH are located at 16q24.2 and possibly at 16q21. Such a high frequency of LOH at a preinvasive stage of breast cancer suggests that a candidate tumor suppressor gene or genes at the 16q23.3-q24.1 location may play an important role in breast carcinogenesis. Further studies are necessary to identify the gene or genes of interest.

## References

1. Marshall, E. Search for a killer: focus shifts from fat to hormones. *Science* (Washington DC), 259: 618-621, 1993.
2. Devilee, P., and Cornelisse, C. J. Somatic genetic changes in human breast cancer. *Biochem. Biophys. Acta*, 1198: 113-130, 1994.
3. Page, D. L., and Dupont, W. D. Anatomic markers of human premalignancy and risk of breast cancer. *Cancer (Phila.)*, 66: 1326-1335, 1990.
4. Cavenee, W. K., Koufos, A., and Hansen, M. F. Recessive mutant genes predisposing to human cancer. *Mutat. Res.*, 168: 3-14, 1986.
5. Chen, T., Dhingra, K., Sahin, A., Sneige, N., Hortobagyi, G., and Aldaz, C. M. Technical approach for the study of the genetic evolution of breast cancer from paraffin-embedded tissue sections. *Breast Cancer Res. Treat.*, 39: 177-185, 1996.
6. Aldaz, C. M., Chen, T., Sahin, A., Cunningham, J., and Bondy, M. Comparative allelotyping of *in situ* and invasive human breast cancer: high frequency of microsatellite instability in lobular breast carcinomas. *Cancer Res.*, 55: 3976-3981, 1995.
7. Lindblom, A., Rotstein, S., Skoog, L., Nordenskjold, M., and Larsson, C. Deletions on chromosome 16 in primary familial breast carcinomas are associated with development of distant metastases. *Cancer Res.*, 53: 3703-3711, 1993.
8. Sato, T., Tanigami, A., Yamakawa, K., Akiyama, F., Kasumi, F., Sakamoto, G., and Nakamura, Y. Allelotyping of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. *Cancer Res.*, 50: 7184-7189, 1990.
9. Tsuda, H., Callen, D. F., Fukutomi, T., Nakamura, Y., and Hirohashi, S. Allele loss on chromosome 16q24.2-qter occurs frequently in breast cancer irrespectively of differences in phenotype and extent of spread. *Cancer Res.*, 54: 513-517, 1994.
10. Cleton-Jansen, A. M., Moerland, E. W., Kuipers-Dijkshoorn, N. J., Callen, D. F., Sutherland, G. R., Hansen, B., Devilee, P., and Cornelisse, C. J. At least two different regions are involved in allelic imbalance on chromosome arm 16q in breast cancer. *Genes Chromosomes & Cancer*, 9: 101-107, 1994.
11. Carter, B. S., Ewing, C. M., Ward, W. S., Treiger, B. F., Aalders, T. W., Schalken, J. K., Epstein, J. I., and Isaacs, W. B. Allelic loss of chromosomes 16q and 10q in human prostate cancer. *Proc. Natl. Acad. Sci. USA*, 87: 8751-8755, 1990.
12. Nishida, N., Fukuda, Y., Kokuryo, H., Sadamoto, T., Isowa, G., Honda, K., Yamaoka, Y., Ikenaga, M., Imura, H., and Ishizaki, K. Accumulation of allelic loss on arms of chromosomes 13q, 16q, and 17p in the advanced stages of human hepatocellular carcinoma. *Int. J. Cancer*, 51: 862-868, 1992.
13. Maw, M. A., Grundy, P. E., Millow, L. J., Eccles, M. R., Dunn, R. S., Smith, P. J., Feinberg, A. P., Law, D. J., Paterson, M. C., Telzerow, P. E., Callen, D. F., Thompson, A. D., Richards, R. I., and Reeve, A. E. A third Wilms'-tumor locus on chromosome 16q. *Cancer Res.*, 52: 3094-3098, 1992.
14. Hudson, T. J., Stein, L. D., and Gerety, S. S., Ma, J., Castle, A. B., Silva, J., Slonim, D. K., Baptista, R., Kruglyak, L., Xy, S-H., Hu, X., Colbert, A. M. E., Rosenberg, C., Reeve-Daly, M. P., Rozen, S., Hui, L., Wu, X., Vestergaard, C., Wilson, K. M., Bae, J. S., Maitra, S., Ganiatsas, S., Evans, C. A., DeAngelis, M. M., Ingalls, K. A., Nahf, R. W., Horton, L. T., Jr., Anderson, M. O., Collymore, A. J., Ye, W., Kouyoumjian, V., Zemsteva, I. S., Tam, J., Devine, R., Courtney, D. F., Renaud, M. T., Nguyen, H., O'Connor, T. J., Fizames, C., Fauré, S., Gyapay, G., Dib, C., Morissette, J., Orlin, J. B., Birren, B. W., Goodman, N., Weissenbach, J., Hawkins, T. L., Foote, S., Page, D. C., and Lander, E. S. An STS-based map of the human genome. *Science* (Washington DC), 270: 1945-1954, 1995.
15. Zhuang, Z., Merino, M. J., Chuaqui, R., Liotta, L. A., and Emmert-Buck, M. R. Identical allelic loss on chromosome 11q13 in microdissected *in situ* and invasive human breast cancer. *Cancer Res.*, 55: 467-471, 1995.
16. O'Connell, P., Pekkel, V., Fuqua, S., Osborne, C. K., and Allred, D. C. Molecular genetics studies of early breast cancer evolution. *Breast Cancer Res. Treat.*, 32: 5-12, 1994.
17. Radford, D. M., Phillips, N. J., Fair, K. L., Ritter, J. H., Holt, M., and Donis-Keller, H. Allelic loss and the progression of breast cancer. *Cancer Res.*, 55: 5180-5183, 1995.
18. Dutrillaux, B., Gerbault-Seureau, M., and Zafrani, B. Characterization of chromosomal anomalies in human breast cancer: comparison of 30 paradigm cases with few chromosome changes. *Cancer Genet. Cytogenet.*, 49: 203-217, 1990.
19. Pandis, N., Heim, S., Bardi, G., Idvall, I., Mandahl, N., and Mitelman, F. Whole-arm t(1;16) and i(1q) as sole anomalies identify gain of 1q as a primary chromosomal abnormality in breast cancer. *Genes Chromosomes & Cancer*, 5: 235-238, 1992.
20. Radford, D. M., Fair, K. L., Phillips, N. J., Ritter, J. H., Steinbrueck, T., Holt, M. S., and Donis-Keller, H. Allelotyping of ductal carcinoma *in situ* of the breast: deletion of loci on 8p, 13q, 16q, 17p, and 17q. *Cancer Res.*, 55: 3399-3405, 1995.

***Advances in Brief*****Analysis of Telomerase Activity Levels in Breast Cancer: Positive Detection at the *in Situ* Breast Carcinoma Stage<sup>1</sup>**

Andrzej K. Bednarek, Aysegul Sahin,  
Andrew J. Brenner, Dennis A. Johnston,  
and C. Marcelo Aldaz<sup>2</sup>

Department of Carcinogenesis, University of Texas M. D. Anderson Cancer Center, Science Park–Research Division, Smithville, Texas 78957 [A. K. B., A. J. B., C. M. A.], and Departments of Pathology [A. S.] and Biomathematics [D. A. J.], University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

**Abstract**

Telomerase activity has been implicated to be associated with most human malignant tumors, including breast cancer. To evaluate possible associations with well-known prognostic factors in breast cancer, we performed a semi-quantitative analysis of telomerase activity levels using the very sensitive PCR-mediated telomeric repeat amplification protocol. Telomerase activity was detected in 99 of 104 breast cancer samples analyzed (95.2%), whereas no activity was detected in 10 of 10 adjacent nonmalignant breast tissues. Analysis of five breast fibroadenoma samples revealed telomerase activity in one (20%). In contrast to previous observations, we observed that 100% of stage I breast tumors were positive for telomerase activity. More interestingly, we detected telomerase activity in six of six ductal carcinoma *in situ* samples (*i.e.*, stage 0). In our semiquantitative analysis of levels of enzymatic activity, we found no statistically significant correlation at the  $P < 0.05$  level between telomerase levels and lymph node metastasis status, estrogen and progesterone receptor status, tumor size, S-phase fraction, and ploidy. The only statistically significant correlation was found with patient age ( $\rho = -0.3$ ;  $P = 0.03$ ). We observed no statistically significant difference in the telomerase activity levels of early tumors (stages 0 and I) *versus* more advanced lesions (stages II to IV). Nevertheless, stage IV tumors displayed a tendency for higher telomerase activity levels. In summary, no clear association was observed between telomerase levels and known breast cancer prognostic indicators. However, telomerase detection by the telomeric repeat amplification protocol method, due to its high sensitivity, may be of value in early breast cancer diagnosis and detection, because our data indicate that

telomerase reactivation appears to constitute a relatively early event in breast carcinogenesis.

**Introduction**

The specialized synthesis of telomeric repeats, TTAGGG in vertebrates, was demonstrated to be performed by the ribonucleoprotein telomerase using its RNA component as a template (1–3). It has been postulated that telomerase activity is associated with acquisition of an immortal phenotype *in vitro*, and it was shown that most immortal cell lines express this enzyme (reviewed in Ref. 4). Using a very sensitive PCR-based TRAP assay,<sup>3</sup> it has been demonstrated that most human adult somatic tissues do not show evidence of active telomerase (5). Activity of this enzyme, however, has been observed in germ-line cells, bone marrow, activated peripheral blood lymphocytes, and possibly stem cells (reviewed in Ref. 4). Interestingly, telomerase activity was detected in numerous human cancer types, suggesting that tumor cells may need reactivation of this enzyme to remain viable (4, 6–14).

Breast cancer is one of the tumor types in which telomerase activity has been demonstrated (12). Furthermore, it has been suggested recently that telomerase detection could have prognostic implications in breast cancer (5, 12). Exactly when in the process of breast carcinogenesis reactivation of telomerase occurs remains to be determined.

Although numerous somatic mutations affecting various genes have been described in sporadic breast cancer, it still remains to be determined which anomalies could be considered causative of breast carcinogenesis. Certain specific aberrations, such as ERBB2 or EGFR amplification and overexpression and P53 mutation, have been postulated and explored as of possible aid in determining breast cancer prognosis (15–18).

In this report, we analyze possible associations of telomerase with well-known prognostic factors in breast cancer to further evaluate whether telomerase detection and quantification could have potential impact on breast cancer prognosis.

**Materials and Methods**

Unselected breast cancer samples were obtained from the Cooperative Human Tissue Network (about one-half of the samples) and from the Department of Pathology, University of Texas M. D. Anderson Cancer Center. Breast cancer samples and adjacent nonmalignant breast tissues were obtained from 104 patients, frozen, and stored at  $-80^{\circ}\text{C}$  until use. We also analyzed a group of five breast fibroadenomas obtained from Cooperative Human Tissue Network. For most of the breast cancer samples, information about estrogen and progesterone

Received 7/10/96; revised 10/9/96; accepted 10/23/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Supported in part by Department of Defense Breast Cancer U. S. Army Grant DAMD17-94-J-4078 (to C. M. A.).

<sup>2</sup> To whom requests for reprints should be addressed. Phone: (512) 237-2403; Fax: (512) 237-2475.

<sup>3</sup> The abbreviations used are: TRAP, telomeric repeat amplification protocol; ITAS, internal telomerase assay standard; DCIS, ductal carcinoma *in situ*.

receptor status, lymph node metastasis status, S-phase, ploidy, tumor stage, and age of patients were available. Tumor staging was performed according to the guidelines of the American Joint Committee on Cancer (19).

The TRAP assay allows the detection of *in vitro* telomerase products (5, 20). We used a modified version of this assay. In brief, telomerase adds TTAGGG repeats to the 3' end of TS primer (5'-AATCCGTCGAGCAGAGTT-3'; Refs. 5 and 21). The number and amount of the repeats added is dependent upon telomerase activity. In a second step, telomerase products are amplified using the CX primer (5'-CCCTTACCCCTTACCC-TACCTAA-3') and Taq DNA polymerase. As positive control, a cell extract from a sample with known telomerase activity (a rat mammary tumor line) was used. As negative control, lysis buffer was substituted for cell extract (20). To compare the level of telomerase activity in different tumor samples, we used a semiquantitative analysis based on the use of an internal standard (ITAS), which amplifies from the same primers (22). This internal standard, which consists of a 150-bp DNA product, allows identification of false-negative tumor samples that could contain Taq polymerase inhibitors.

Cell extracts were obtained from 10–50-mg samples of the tumors, and telomerase assay was performed according to a method described previously (5, 20), with minor modifications (22, 23). In a standard procedure, we used 2  $\mu$ l of tissue extract (protein concentration, 0.5  $\mu$ g/ $\mu$ l) per assay. The CX primer, ITAS, and Taq DNA polymerase (7 units/assay) were added to each sample at a "hot start" after 5 min incubation at 90°C. Because telomerase has an RNA component, 5  $\mu$ l of the tumor cell extract from the same samples and the positive cell extract were incubated with 1  $\mu$ l of RNase A (1 mg/ml) as an additional experimental control.

Aliquots (10  $\mu$ l) of the PCR mixture were analyzed on 8% nondenaturing, 0.4-mm acrylamide gels (20  $\times$  40 cm) run in 0.5 $\times$  TBE buffer until the xylene cyanol had migrated 17 cm from the origin. The gels were then dried and exposed for 20 h to hyperfilm MP films (Amersham Corp., Arlington Heights, IL). Following autoradiography, each gel was analyzed after overnight exposure using a Molecular Dynamics PhosphorImager (Sunnyvale, CA). This scan was used to perform the measurements of the telomerase ladder amplification intensity. Thus, area integration of all peaks (except the first band from the bottom) were normalized to the signal from the internal standard and then, after background subtraction, expressed as relative to the positive control signal that was run with each experiment. The first band from the bottom was not included, because it usually incorporates background from primer-dimer formation (8, 13). The method described is only semiquantitative, but it is sufficient for the comparative analysis of the tumors relative to the same positive-control cell extract.

Analysis of the levels of telomerase activity and other clinico-pathological characteristics was performed using non-parametric Spearman rank correlation and *t* test.

## Results and Discussion

To confirm the linearity of the TRAP assay, we performed a dilution experiment of the telomerase-positive breast cancer cell line MDA-MB-157 (Fig. 1). The activity of the telomerase

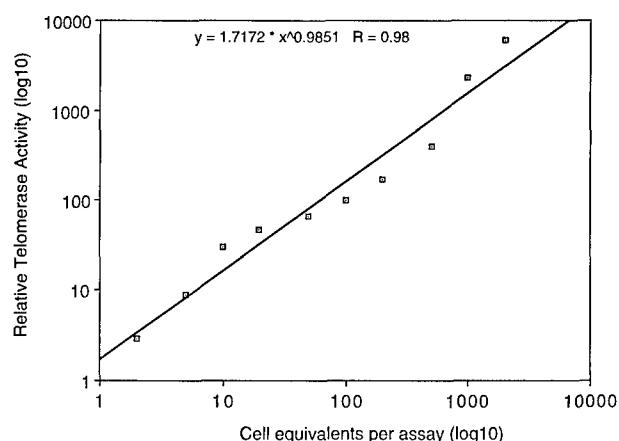


Fig. 1 Quantification of telomerase activity from breast cancer cell line MDA-MB-157. The activity level was calculated by summation of integrated areas of the telomerase ladder and normalized to internal standard (ITAS). After background subtraction, levels were expressed as relative to a 100-cell equivalent sample. The *solid line* was computer fitted according to a calculated algorithm.

ladder was normalized to that of the internal standard (ITAS; Ref. 22), expressing the results relative to the activity found in the 100-cell equivalent sample. Linearity of activity with cell number was observed (Fig. 1), in agreement with observations published previously (22).

Telomerase activity was then analyzed and detected in 99 of the 104 breast cancer samples (95%). It was undetectable in 10 of 10 adjacent nonmalignant breast samples (Fig. 2). As expected, the enzymatic activity detected is abolished if samples are pretreated with RNase A (Fig. 2).

Interestingly, we detected telomerase activity in six of six DCIS samples analyzed (Fig. 3A; stage 0 in Fig. 4A). Four of these DCIS samples were classified as high nuclear grade and showed high telomerase activity, whereas the two samples classified as low nuclear grade showed very weak telomerase activity (Fig. 3A). These findings suggest that the reactivation of telomerase activity occurs relative early in breast carcinogenesis. We also analyzed a small group of breast fibroadenomas for telomerase activity and observed one positive tumor of five tested (20%).

Several clinical, histological, and biological indicators of prognosis are commonly used to determine the therapeutic management of breast cancer patients. Marker combinations are usually more accurate than single markers (18, 24). A major goal of our study was to evaluate the role of telomerase detection as a possible additional prognostic indicator. Our overall incidence of telomerase-positive breast cancer samples (95.2%) is similar to that reported previously by Hiyama *et al.* (Ref. 12; 93%). However, whereas Hiyama *et al.* (12) found 68% of stage I breast carcinomas to be positive for telomerase activity, we found 100% of the 17 stage I breast carcinomas to be positive. The few tumors negative for telomerase activity were found to be advanced-stage (II and III) rather than early-stage tumors, as can be observed in Fig. 4A, in which breast cancer samples are grouped by tumor stage. Furthermore, we did not find any correlation between the very few tumors (five samples) found to

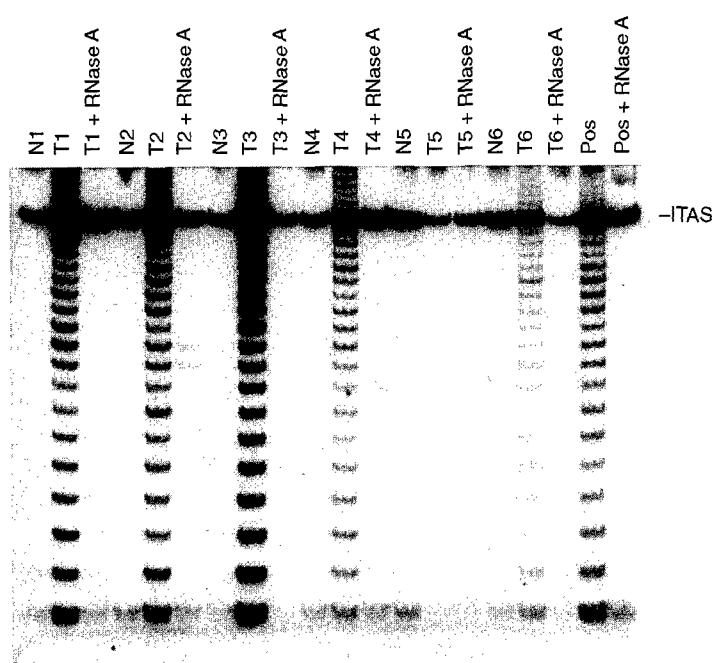
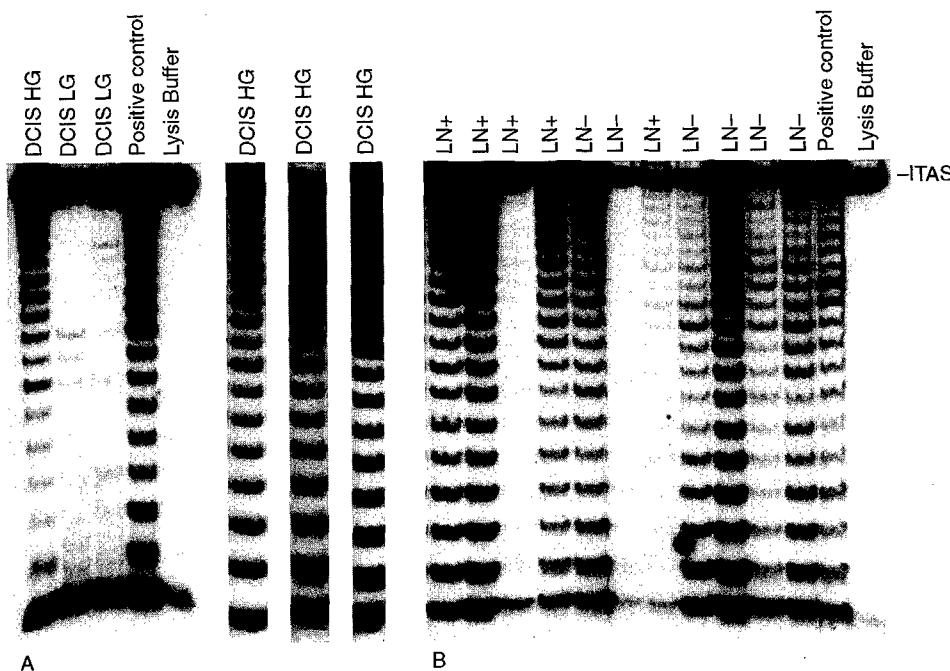


Fig. 2 TRAP assay from human breast cancers (*T*) and matching adjacent nonmalignant tissues (*N*) with internal standard (*ITAS*). To show assay specificity, tumor samples were RNase A pretreated (+RNase A).



**Fig. 3** Detection of telomerase activity in human breast cancers. **A**, telomerase activity in DCIS (*LG*, low nuclear grade; *HG*, high nuclear grade). **B**, activity in lymph node metastasis negative (*LN-*) and positive (*LN+*) tumors. Note that lymph node-negative tumors have the same activity levels as lymph node-positive tumors (*ITAS*, internal standard).

be negative for telomerase activity and any of the known prognostic indicators.

In our semiquantitative analysis of levels of enzymatic activity, we found no statistically significant correlation at the  $P < 0.05$  level between telomerase levels and tumor size, lymph node metastasis, estrogen and progesterone receptor status, S-phase fraction, and ploidy, (Fig. 4 and Table 1). Axillary lymph node status is generally accepted as one of the

best prognostic indicators for breast cancer recurrence (24). We observed as much telomerase activity in lymph node-negative tumors as in tumors positive for lymph node metastasis (Fig. 4E). Interestingly, we only found statistically significant correlation with patient age (Fig. 4C; Table 1). It appears that levels of telomerase activity are higher in tumors from younger patients. However, although  $P = 0.03$ , the Spearman rank correlation analysis indicates that since the

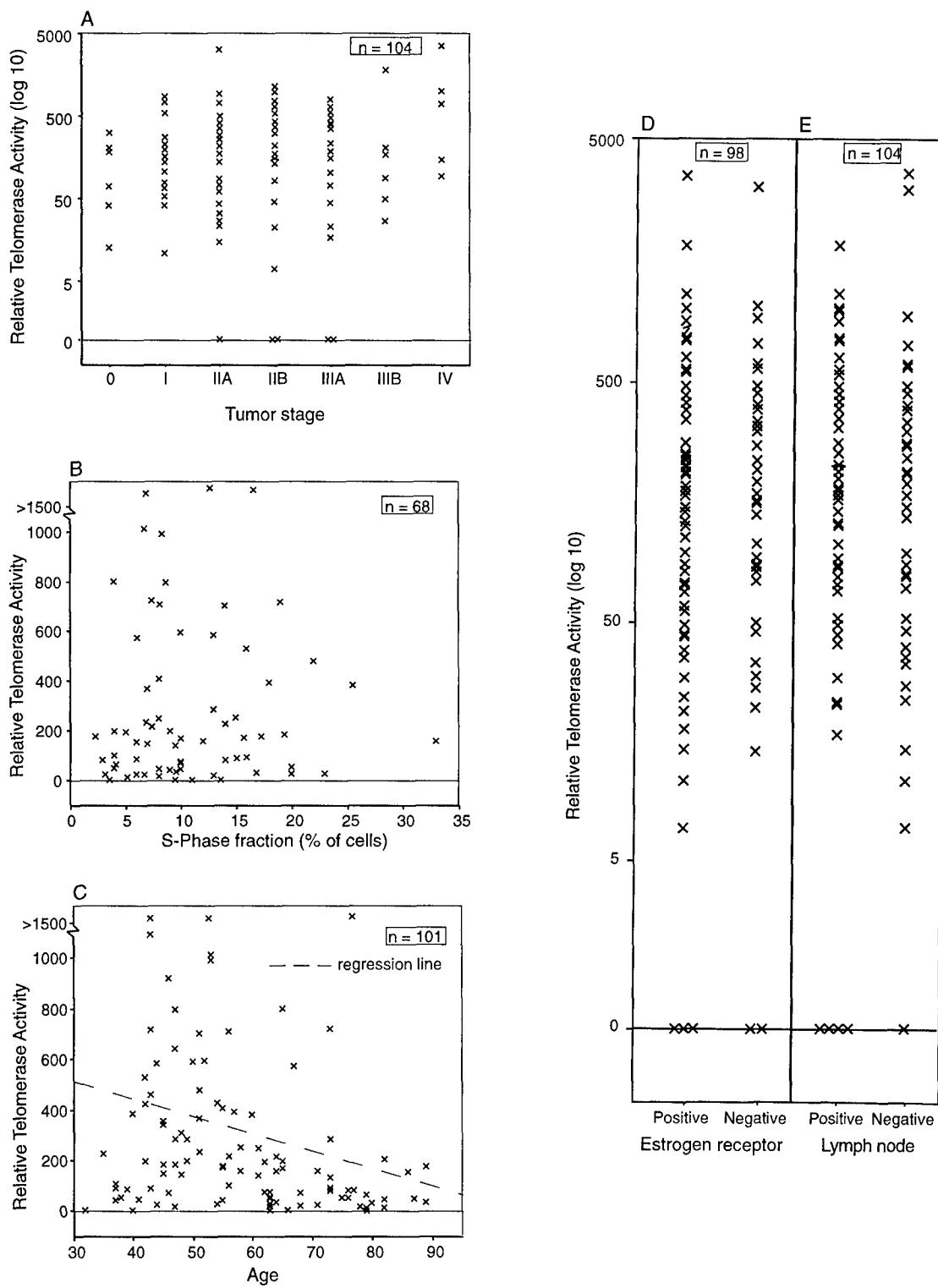


Fig. 4 Comparison of relative telomerase activity levels to prognostic indicators in breast cancer.

rho value is  $-0.3$  (Table 1); approximately only 9% of the variability in telomerase levels is explained by age. As mentioned earlier, we observed that all stage 0 and stage I tumors express telomerase activity. We did not observe a statistically

significant difference in telomerase activity levels of early tumors (stages 0 and I) *versus* more advanced lesions (Fig. 4A and Table 1). However, as can be observed in Fig. 4A and Table 1, stage IV tumors appear to show a tendency for

Table 1 Statistical analysis of telomerase activity levels

	Age	Ploidy	Size	S-phase
rho (Spearman rank correlation)	-0.3	0.02	0.06	0.1
P	0.03	0.88	0.56	0.39
Comparison of breast tumor status <sup>a</sup>				P(t-test)
ER+ (318 ± 67) versus ER- (333 ± 91)				
PR+ (276 ± 60) versus PR- (405 ± 101)				
ER+/PR+ (277 ± 68) versus ER-/PR- (345 ± 111)				
LN+ (319 ± 49) versus LN- (371 ± 113)				
Stages 0 and I (249 ± 52) versus II-IV (367 ± 70)				
Stages 0-IIIB (290 ± 45) versus IV (1083 ± 623)				

<sup>a</sup> ER+, estrogen receptor positive; ER-, estrogen receptor negative; PR+, progesterone receptor positive; PR-, progesterone receptor negative; LN, lymph node. Values in parentheses represent mean ± SE. The analysis was performed using StatView 4.0 (Abacus Concepts).

higher telomerase levels. The small number of stage IV tumors precludes further speculation on this observation.

Although patient follow-up was not analyzed in our study, the lack of a clear association between telomerase activity levels and proven prognostic indicators in breast cancer indicates that analysis of the levels of this enzyme by means of the PCR-mediated TRAP assay in human breast cancer may have limited value as a prognostic tool. This appears in contrast to preliminary observations by another laboratory (25). It is unclear at this point whether the hypothesis that a worse prognosis should correlate with higher telomerase levels is incorrect, or alternatively that the lack of correlation observed is the result of the methodological approach. It is then possible that future development of non-PCR-mediated methodologies for measurement of telomerase activity may be better suited for studies of prognosis. On the other hand, because we detected telomerase activity at preinvasive stages of breast cancer, the highly sensitive TRAP assay may be of value in early breast cancer detection and diagnosis, as recently suggested by Hiyama *et al.* (26). Our findings are in agreement with observations of other neoplasias in which telomerase activity was detected at preinvasive stages of tumor development (13, 14). For instance, telomerase activity has been demonstrated in premalignant prostatic hyperplasias (13), and recently telomerase activity has been also observed in colon adenomas (14). We have also reported a significant increase in telomerase activity at premalignant stages using a mouse multistage carcinogenesis model (23).

As mentioned previously, we also observed that one of five breast fibroadenomas analyzed showed telomerase activity. This incidence appears lower than that reported previously (45%; Ref. 12). Despite the difference in incidence, both studies indicate that some breast fibroadenomas show reactivation of telomerase. It is unclear at this point the putative role for telomerase reactivation in this benign neoplasia.

In summary, telomerase activity was observed in almost all breast cancer samples, regardless of tumor stage. No telomerase activity was detected in the normal breast samples tested, and telomerase activity was detected in 20% of fibroadenomas. No correlation was found between telomerase detection or level of activity and known breast cancer prognostic indicators, which appears to limit the potential value of evaluating the level of this putative biomarker in managing patients with invasive breast cancer. Nevertheless, the final conclusion on this issue will only

be resolved after considering the correlation between patient outcome and telomerase levels. Probably the most important conclusion from our study is that telomerase reactivation appears to be an early event in breast carcinogenesis. This factor highlights the potential for using telomerase detection as a possible aid in early tumor detection.

### Acknowledgments

We thank Dr. Jerry Shay for the ITAS standard samples.

### Note Added in Proof

While this manuscript was being reviewed, Sugino *et al.* reported similar conclusions on the potential value of telomerase detection as an early diagnostic marker in breast cancer rather than as a prognostic indicator (Sugino, T., Yoshida, K., Bolodeoku, J., Tahara, H., Buley, I., Manek, S., Wells, C., Goodison, S., Ide, T., Suzuki, T., Tahara, E., and Tarin, D. Telomerase activity in human breast cancer and benign breast lesions: diagnostic applications in clinical specimens, including fine needle aspirates. *Int. J. Cancer*, 69: 301–306, 1996).

### References

- Greider, C. W., and Blackburn, E. H. The telomere terminal transferase of *tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity. *Cell*, 51: 887–898, 1987.
- Morin, G. B. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell*, 59: 521–529, 1989.
- Feng, J., Funk, W. D., Wang, S-S., Weinrich, S. L., Avilion, A. A., Chiu, C-P., Adams, R. R., Chang, E., Allsopp, R. C., Yu, J., Le, S., West, M. D., Harley, C. B., Andrews, W. H., Greider, C. W., and Villeponteau, B. The RNA component of human telomerase. *Science* (Washington DC), 269: 1236–1241, 1995.
- Holt, S. E., Shay, J. W., and Wright, W. E. Refining the telomere-telomerase hypothesis of aging and cancer. *Nat. Biotechnol.*, 14: 836–839, 1996.
- Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L. C., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. Specific association of human telomerase activity with immortal cells and cancer. *Science* (Washington DC), 266: 2011–2015, 1994.
- Counter, C. M., Hirte, H. W., Bacchetti, S., and Harley, C. B. Telomerase activity in human ovarian carcinoma. *Proc. Natl. Acad. Sci. USA*, 91: 2900–2904, 1994.
- Hiyama, E., Yokoyama, T., Tatsumoto, N., Hiyama, K., Imamura, Y., Murakami, Y., Kodama, T., Piatyszek, M. A., Shay, J. W., and

Matsuura, Y. Telomerase activity in gastric cancer. *Cancer Res.*, **55**: 3258–3262, 1995.

8. Chadeneau, C., Hay, K., Hirte, H. W., Gallinger, S., and Bacchetti, S. Telomerase activity associated with acquisition of malignancy in human colorectal cancer. *Cancer Res.*, **55**: 2533–2536, 1995.

9. Hiyama, E., Hiyama, K., Yokoyama, T., Matsuura, Y., Piatyszek, M. A., and Shay, J. W. Correlating telomerase activity levels with human neuroblastoma outcomes. *Nat. Med.*, **1**: 249–257, 1995.

10. Hiyama, K., Hiyama, E., Ishioka, S., Yamakido, M., Inai, K., Gazdar, A. F., Piatyszek, M. A., and Shay, J. W. Telomerase activity in small-cell and non-small-cell lung cancers. *J. Natl. Cancer Inst.*, **87**: 859–902, 1995.

11. Avilion, A. A., Piatyszek, M. A., Gupta, J., Shay, J. W., Bacchetti, S., and Greider, C. W. Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues. *Cancer Res.*, **56**: 645–650, 1996.

12. Hiyama, E., Gollahon, L., Kataoka, T., Kuroi, K., Yokoyama, T., Gazdar, A. F., Hiyama, K., Piatyszek, M. A., and Shay, J. W. Telomerase activity in human breast tumors. *J. Natl. Cancer Inst.*, **88**: 116–122, 1996.

13. Sommerfeld, H.-J., Meeker, A. K., Piatyszek, M. A., Bova, G. S., and Shay, J. W. Telomerase activity: a prevalent marker of malignant human prostate tissue. *Cancer Res.*, **56**: 218–222, 1996.

14. Tahara, H., Endo, H., Maeda, S., Nakanishi, T., Nagai, N., Ide, T., and Tahara, E. Telomerase activity in various precancerous and cancer tissues. *Proc. Am. Assoc. Cancer Res.*, **37**: 559, 1996.

15. Caleffi, M., Teague, M. W., Jensen, R. A., Vnencak-Jones, C. L., Dupont, W. D., and Parl, F. F. *p53* gene mutations and steroid receptor status in breast cancer. *Cancer (Phila.)*, **73**: 2147–2156, 1994.

16. Ravdin, P. M., and Chamness, G. C. The *c-erbB-2* proto-oncogene as a prognostic and predictive marker in breast cancer: a paradigm for the development of other macromolecular markers—a review. *Gene (Amst.)*, **159**: 19–27, 1995.

17. Nakopoulou, L. L., and Alexiadou, A. Prognostic significance of the co-expression of *p53* and *c-erbB-2* proteins in breast cancer. *J. Pathol.*, **179**: 31–38, 1996.

18. Scholl, S., Bieche, I., Pallud, C., Champeme, M. H., Beuvon, F., and Hacene, K. Relevance of multiple biological parameters in breast cancer prognosis. *Breast*, **5**: 21–30, 1996.

19. Beahrs, O. H., Henson, D. E., Hutter, R. V. P., and Kennedy, B. J. (eds.). *American Joint Committee on Cancer. Manual for Staging of Cancer*, Ed. 4. Philadelphia: J. B. Lippincott Co., 1992.

20. Piatyszek, M. A., Kim, N. W., Weinrich, S. L., Hiyama, K., Hiyama, E., Wright, W. E., and Shay, J. W. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). *Methods Cell Sci.*, **17**: 1–15, 1995.

21. Morin, G. B. Recognition of a chromosome truncation site associated with  $\alpha$ -thalassaemia by human telomerase. *Nature (Lond.)*, **353**: 454–456, 1991.

22. Wright, W. E., Shay, J. W., and Piatyszek, M. A. Modifications of a telomeric repeat amplification protocol (TRAP) results in increased reliability, linearity and sensitivity. *Nucleic Acids Res.*, **23**: 3794–3795, 1995.

23. Bednarek, A., Budunova, I., Slaga, T. J., and Aldaz, C. M. Increased telomerase activity in mouse skin premalignant progression. *Cancer Res.*, **55**: 4566–4569, 1995.

24. Donegan, W. L. Prognostic factors: stage and receptor status in breast cancer. *Cancer (Phila.) (Suppl.)*, **70**: 1755–1764, 1992.

25. Kim, N. W., Levitt, D., Huang, G., Wu, F., Osborne, K., and Clark, G. Correlation of telomerase with prognostic indicators of breast cancer. *Proc. Am. Assoc. Cancer Res.*, **37**: 562, 1996.

26. Hiyama, E., Gollahon, L., Kataoka, T., Kuroi, K., Yokoyama, T., Gazdar, A. F., Hiyama, K., Piatyszek, M. A., and Shay, J. W. Telomerase activity in human breast tumors. *J. Natl. Cancer Inst.*, **88**: 839–840, 1996.

PROGRESS IN CLINICAL  
AND BIOLOGICAL RESEARCH  
VOLUME 396

---

**ETIOLOGY OF BREAST  
AND GYNECOLOGICAL  
CANCERS**

Editors: C. Marcelo Aldaz  
Michael N. Gould  
John McLachlan  
Thomas J. Slaga

---



**WILEY-LISS**

---

---

# ETIOLOGY OF BREAST AND GYNECOLOGICAL CANCERS

Proceedings of the Ninth International Conference on Carcinogenesis and Risk Assessment, Held in Austin, Texas, November 29 – December 2, 1995

---

---

Editors

**C. Marcelo Aldaz**

University of Texas  
M.D. Anderson Cancer Center  
Science Park – Research Division  
Smithville, Texas

**Michael N. Gould**

Comprehensive Cancer Center  
University of Wisconsin  
Madison, Wisconsin

**John McLachlan**

Department of Pharmacology  
Center for Bioenvironmental Research  
Tulane University  
New Orleans, Louisiana

**Thomas J. Slaga**

University of Texas  
M.D. Anderson Cancer Center  
Science Park – Research Division  
Smithville, Texas



A JOHN WILEY & SONS, INC., PUBLICATION  
New York • Chichester • Weinheim • Brisbane • Singapore • Toronto

## THE GENETICS OF SPORADIC BREAST CANCER

Andrew J. Brenner and C. Marcelo Aldaz

Department of Carcinogenesis, University of Texas M.D. Anderson Cancer  
Center, Science Park-Research Division, Smithville, Texas 78957

### INTRODUCTION

Breast cancer which affects as many as one in eight women, is the most common malignancy of women in the industrialized countries of the Western hemisphere (Feuer, 1993; Boring, 1994). To expedite the development of new treatment strategies, increased emphasis has been placed on understanding the cellular and molecular events that lead to malignancies of the breast. Over the past few years, numerous advances have been made in the elucidation and characterization of genes whose mutation predisposes individuals to risk of developing familial breast cancer. These genes include the recently cloned *BRCA1* and *BRCA2* (Miki et al., 1994; Wooster et al., 1995). However, while these genes have been shown to be frequently affected in inheritable forms of breast cancer, there is yet no conclusive evidence to suggest that these genes are also responsible for sporadic breast cancer which accounts for approximately 90% of breast cancer cases. To provide insight into the aberrations responsible for the genesis of sporadic breast cancer, ongoing work is attempting to identify genomic regions frequently affected. In this chapter, we will focus on the known somatic genetic aberrations of sporadic breast cancer.

### CYTOGENETICS OF BREAST CANCER

Numerous attempts have been made toward characterization of aberrations at the chromosomal level in breast cancer. However, as is the case with other solid tumors of epithelial origin, it has been difficult to discern any characteristic primary cytogenetic changes among the large number of apparently random alterations. This is due to the inherent difficulties in obtaining high-quality metaphases from solid tumors as well as their characteristic clonal heterogeneity. In addition, of the tumors that have been karyotyped, the vast majority are of the more advanced invasive stages, since the less advanced "in situ" carcinomas tend to be much smaller, thereby making it even more difficult to obtain high-quality metaphase cells. Nevertheless, several genetic changes with a relatively higher prevalence have been identified. Overall, the most frequent tend to be numerical alterations

of whole chromosome copy number including trisomies of 7 and 18 and monosomies of 6, 8, 11, 13, 16, 17, 22, and X (reviewed by Devilee and Cornelisse, 1994). The most common aberrations in near-diploid tumors without metastases are loss of chromosomes 17 and 19, trisomy of chromosome 7, and overrepresentation of 1q, 3q, and 6p (Thompson et al., 1993). Structural alterations include terminal deletions and unbalanced nonreciprocal translocations, most frequently involving chromosomes 1, 6, and 16q. Breakpoints of structural abnormalities cluster to several segments, including 1p22-q11, 3p11, 6p11-13, 7p11-q11, 8p11-q11, 16q, and 19q13 (Thompson et al., 1993). Tumors from patients with metastatic breast carcinoma display a different pattern of abnormalities with structural alterations and numerous numerical alterations affecting various chromosomes (Trent et al., 1993).

A recently developed technique, comparative genomic hybridization (CGH), allows analyses of chromosome copy number abnormalities involving segments of at least 10 Mb (Kallioniemi et al., 1992A). Since CGH involves hybridizing differentially labeled genomic DNA from a tumor and a normal cell population to the same normal metaphase, it circumvents some of the difficulties encountered in conventional karyotyping. Through such analyses, nearly every tumor analyzed revealed increased or decreased DNA sequence copy number (Kallioniemi et al., 1994). The most common regions of increased copy number in breast cancer as determined by CGH include 1q, 8q, 17q22-24, and 20q13. Increased copy number at 17q was previously determined through gene fluorescence *in situ* hybridization (FISH) analysis to be 50 to 100-fold amplification of *ERBB2* (Kallioniemi et al., 1992B), a gene known to be overexpressed and amplified in a considerable percentage of breast cancers. Amplifications at region 8q would analogously be *MYC*, another gene known to be overexpressed in some breast cancers (Shiu, Watson, and Dubik, 1993). No candidate gene has yet been identified for region 20q13 amplification. Regions of decreased DNA copy number were also observed and include 3p, 6q, 8p, 11p, 12q, 13q, and 17p (Gray et al., 1994). For some of these regional losses, candidate genes exist that may be the target of deletion in the progression to a malignant phenotype (Table 1). More specifically, both *RB1* (13q) and *TP53* (17p) have been shown extensively to undergo deletion in a significant fraction of breast cancers (Cox, Chen, and Lee, 1994). Interestingly, when both loss and gain of DNA copy number as determined by CGH were compared with survival data in a series of node negative breast tumors, only copy number losses were significant for recurrence and for overall survival (Isola et al., 1995). However, as is the case with conventional cytogenetics, CGH has yet to reveal any characteristic abnormalities that occur in the majority of breast tumors and which abnormalities if any, could be considered "primary".

## ONCOGENES

The proto-oncogenes encode proteins involved in a cascade of events leading to growth in response to mitogenic factors. Alteration in the normal function of proto-oncogenes, through mutation or increased expression can result in a constant growth stimulus and a constitutive mitogenic response. Aberration of a single allele of an oncogene can be sufficient to lead to altered signal and as such is dominant. In human solid tumors, the most common aberration affecting oncogenes appears to be gene amplification.

Genetic Region	Cytogenetic <sup>1</sup> Finding	CGH <sup>2</sup> Finding	Invasive <sup>3</sup> LOH (%)	DCIS <sup>3</sup> LOH (%)	Possible Targets
1p	-1p		32	8	
1q	+1q	+1q	30	16	
3p		-3p	22	0	
3q			25	0	
4p	-4p		2 <sup>1</sup>	0 <sup>†</sup>	
5p			18 <sup>1</sup>	0 <sup>†</sup>	
5q			13 <sup>1</sup>	0 <sup>†</sup>	
6p	-6	+6p	30	0	
6q	-6, -6q	-6q, +6q	26	8	
7p	+7	+7p	32	32	
7q	+7		25	24	
8p	-8, -8p	-8p	18	10	
8q	-8, +8q	+8q	20	22	<i>MYC</i>
9p	-9p		58	30	<i>p16INK4a</i>
9q			24 <sup>1</sup>	0 <sup>†</sup>	
10p			11 <sup>1</sup>	0 <sup>†</sup>	
10q			15 <sup>1</sup>	0 <sup>†</sup>	
11p	-11, -11p	-11p	28	0	
11q	-11, -11q	+11q	30	12	<i>CCND1</i>
12p			8 <sup>1</sup>	0 <sup>†</sup>	
12q		+12q	4 <sup>1</sup>	0 <sup>†</sup>	
13q	-13	-13q	30	18	<i>RBI, BRCA2, Brush</i>
16p	-16		40	0	
16q	-16, -16q		48	27	<i>CDH1?</i>
17p	-17, -17p	-17p	57	33	<i>TP53</i>
17q	-17	+17q	36	31	<i>BRCA1, NME1, ERBB2</i>
18p			25	0	
18q			48	12	
19p			18 <sup>1</sup>	0 <sup>†</sup>	
19q		+19q	14 <sup>1</sup>	0 <sup>†</sup>	
20q		+20q13	17 <sup>1</sup>	6 <sup>†</sup>	
21q			17 <sup>1</sup>	5 <sup>†</sup>	
22q	-22		36	0	
Xp	-X		22 <sup>1</sup>		
Xq	-X		8 <sup>1</sup>		

Table 1. Summary of genetic aberrations affecting sporadic breast cancer (<sup>1</sup> Devilee and Cornelisse [1994], Thompson et. al. [1993]; <sup>2</sup> Gray et. al. [1994]; <sup>3</sup> Aldaz et. al. [1995]; <sup>†</sup> Radford et. al. [1995]).

Current data suggests of the numerous oncogenes described to date, that only a few may have a role in breast tumorigenesis. Of these, *ERBB2* remains the oncogene most studied in breast cancer. Also known as *HER2* or *neu*, *ERBB2* encodes a tyrosine kinase growth factor receptor with high homology to epidermal growth factor receptor (43% in the extracellular domain and 82% in the tyrosine kinase domain; Coussens et al., 1985; Jardines et al., 1993). However, while these two receptors share homology, their ligand specificity is distinct since neither epidermal growth factor nor transforming growth factor- $\alpha$  bind *ERBB2*. Activation or overexpression of *ERBB2* in transgenic mice results in the genesis of mammary tumors (Bouchard et al., 1989; Muller et al., 1988). As mentioned previously, FISH analysis has shown that *ERBB2* is amplified. Prior analysis by other means had shown amplification of *ERBB2* in 25-30% of breast cancers with concomitant overexpression, implicating its involvement in breast tumorigenesis (Berger et al., 1988; Zhou et al., 1987). Early studies reported a prognostic value of *ERBB2* overexpression in node-negative breast cancer. However, more recent studies using larger data sets do not support these early observations and question the prognostic role for *ERBB2* expression in node-positive breast cancer. Expression of *ERBB2* may have value in predicting response to specific therapies, but additional studies are needed to confirm these preliminary findings (reviewed by Ravdin and Chamness, 1995).

Similar to *ERBB2*, the *MYC* gene has been shown to be amplified in approximately 25% of breast carcinomas. Although the functions of *Myc* are not yet clearly understood, *c-Myc* has been shown to heterodimerize with Max (*c-Myc*-associated protein X), positively and negatively regulating the expression of various genes in apoptosis and cell cycle progression (Ryan and Birnie, 1996). Overexpression of *c-Myc* in transgenic mice results in mammary tumors (Muller et al., 1988), and amplification of *c-Myc* has been associated with high grade tumors in humans (Varley et al., 1987). However, when lymph node metastases from patients whose primary tumor showed amplification are examined, the metastatic cells do not show amplification, suggesting that amplification occurs before invasion and is not a prerequisite for a metastatic phenotype (Shiu et al., 1993). Of additional interest, *c-Myc* expression is modulated by the presence of estrogen in estrogen-responsive cell lines, and constitutively high *c-Myc* expression is observed in hormone-dependent lines, probably because of increased stability of the transcript (Shiu et al., 1993).

Chromosome region 11q13 has also been reported to be amplified in 15-20% of breast cancers (Lammie and Peters, 1991). The cyclin D1 (*CCND1*) gene, located in the region, is thought to be the target of such amplification. Cyclin D1 is a direct regulator of the cell cycle and is overexpressed in 45% of breast carcinomas, most of which are both estrogen and progesterone receptor positive (Gillet et al., 1994; Bartkova et al., 1994). Studies show that transgenic mice homozygously null for *CCND1* fail to undergo proliferative changes of the mammary epithelium associated with pregnancy, thereby indicating a role for *CCND1* in steroid-induced proliferation of the mammary epithelium (Sicinski et al., 1995). Transgenic mice overexpressing *CCND1* have been shown to develop mammary carcinomas (Wang et al., 1994). Analysis of *CCND1* expression by mRNA *in situ* hybridization has shown a dramatic increase of *CCND1* expression in 76% of low grade carcinoma *in situ*, further suggesting a role for *CCND1* in the tumorigenesis of the breast (Weinstat-Saslow et al., 1995).

## TUMOR SUPPRESSORS AND LOSS OF HETEROZYGOSITY

Although the first tumor suppressor gene, *RBI*, was not identified until 1987, the existence of a genetic element with growth suppressive properties had been shown nearly two decades earlier. When Harris et al. fused normal mouse fibroblasts with highly malignant tumor cells, the resultant hybrids lost all tumorigenic capacity (Harris et al., 1969). Further, passage *in vitro* resulted in segregants that reverted to malignant phenotype upon loss of chromosomes (Harris et al., 1969; Klein et al., 1971). Two years later, Knudson, on the basis of statistical analysis of clinical observations, was the first to suggest retinoblastoma was a cancer caused by two mutational events (Knudson, 1971). In the hereditary form, one mutation was germinal; thus only a single additional somatic mutation was required. This results in early onset and a tendency toward bilateral tumorigenesis. In the sporadic form, both mutations are somatic, resulting in a tendency toward unilaterally and late onset. Comings later suggested that these two mutational events could occur within separate alleles of a regulatory gene (Comings, 1973). Indeed, cytogenetic analysis of retinoblastoma revealed characteristic deletions of the long arm of chromosome 13. Subsequent analysis of this region led to the identification of *RBI* and elucidation of aberrant transcripts encoded from the remaining allele (reviewed by Goodrich and Lee, 1993). Hence, a precedent emerged where inactivation of one allele of a tumor suppressor is accomplished by mutation, leading to the eventual deletion of the remaining normal allele through chromosomal aberrations and loss of heterozygosity (LOH) is thereby observed in the suppressor locus. This precedent is now considered the convention for suppressor inactivation and similar observations have been made for several other suppressive genes (e.g., *APC*, *DCC*, *VHL*, *TP53*; reviewed by Cox, Chen, and Lee, 1994). Further, LOH is considered indirect evidence for the existence of a suppressor gene within the affected region.

### Allelotype of Breast Cancer

Because the mechanisms by which loss of heterozygosity occurs tend to involve large segments of DNA, it is possible to utilize the neighboring genes or known noncoding sequences as indicators to identify deleted regions harboring putative suppressor genes whose loss may be important in the genesis of the tumor. One such genetic marker is naturally occurring simple sequence length polymorphisms (SSLPs). SSLPs consist mainly of dinucleotide repeats, primarily (CA)<sub>n</sub>, which are repeated in tandem at variable number (n) interspersed throughout the genome. To date, more than 5,000 such SSLPs with length polymorphisms of approximately 10-60 repeats, termed polymorphic microsatellites, have been identified (Dib et al., 1996; Litt and Luty, 1989). These polymorphic microsatellites have a mean heterozygosity of 70% at an average interval size of 1.6 cM. Through known linkage maps and comparison to physical maps, it is possible to select highly polymorphic microsatellites at any position within the genome. Further, through PCR amplification of these microsatellites and comparison with normal DNA from the same patient, it is possible to generate a comprehensive map of allelic imbalances (allelotype) occurring in a neoplasm (Weber and May, 1989).

Allelotyping of breast cancer has been reported in numerous studies, and numerous regions of allelic imbalance have been described using microsatellites as well as the older

restriction fragment length polymorphism analysis. As reviewed by Devilee and Cornelisse, compilation of data from more than 30 studies reveals a consensus of imbalances affecting more than 11 chromosome arms at a frequency of more than 25% (Table 1). Chromosome arms 1p, 1q, 3p, 6q, 8p, 11p, 13q, 17q, 18q, and 22q were affected at a frequency of 25-40%, whereas chromosome arms 16q and 17p were affected in more than 50% of tumors (Devilee and Cornelisse, 1994). In addition, chromosome arm 9p, which was not evaluated in these studies most likely because of lack of previous cytogenetic data implicating it, has recently been shown by our laboratory to be affected in 58% of breast carcinomas (Brenner and Aldaz, 1995). The loss of generic material in many of these regions has been corroborated by either CGH or classic cytogenetic data (Devilee and Cornelisse, 1994; Trent et al 1993). Further, some of these regions are known to harbor tumor suppressive genes whose loss has been demonstrated through a variety of techniques, including Southern blot analysis and FISH using gene-specific single-copy probes (Cox, Chen, and Lee, 1994).

While there is overwhelming evidence that these genetic losses occur, inherent difficulties exist in determining the relevance of such losses to breast tumorigenesis. In most cases, the tumors analyzed were of the invasive type and/or advanced stages of progression, leading to the question whether these losses are causative factors of tumorigenesis or consequences of the general genomic instability inherent to tumors. Further, it is possible that certain losses may be selected for in the progression or clonal evolution of a tumor to a more advanced type but not necessary for the genesis of the tumor. Some of these questions could be addressed in part through comparative allelotyping of both noninvasive and invasive tumors.

To address the relative timing and frequency of allelic losses of commonly affected regions in breast cancer, microsatellite length polymorphism analysis was performed in a series of preinvasive ductal carcinomas (DCIS) and invasive ductal and lobular carcinomas (Aldaz et al, 1995). Twenty different loci were examined in each group. As expected, frequencies of regional losses in invasive ductal carcinomas were similar to those in the aforementioned compilation by Devilee and Cornelisse of analyses from more than 30 studies. However, allelotyping of DCIS samples revealed that chromosomal regions 3p, 3q, 6p, 11p, 16p, 18p, 18q, and 22q were not affected by a high frequency of loss, while analyses of these same regions of invasive tumors showed them to be affected in 10-40% of cases (Aldaz et al., 1995). Our findings are in agreement with those of Radford et. al. who examined 61 DCIS samples (Radford et al, 1995). Because allelic losses affecting these regions were not frequently observed at the noninvasive (DCIS) stage it can be concluded that alterations of these regions are late events in breast cancer progression. More importantly, allelic imbalances observed on chromosome arms 7p, 7q, 16q, 17p, and 17q (Aldaz et al., 1995), as well as 9p as reported by others (Fujii et al., 1996), appear to be early abnormalities because they occur in approximately one third of DCIS samples.

Lobular carcinomas constitute approximately 10-15% of all breast cancers (Tavassoli, 1992). Histologically, lobular carcinomas have a very distinctive infiltrative growth pattern and metastatic pattern (Tavassoli, 1992). In addition, patients with invasive lobular carcinoma have been reported to have a higher risk of developing multifocal and contralateral breast cancer than those patients with invasive ductal carcinoma (Silverstein et al., 1994). To determine whether ductal and lobular carcinomas are subject to the same pattern of allelic loss, comparative allelotyping of the two subtypes was also conducted in

our laboratory. Losses of chromosome arms 1p, 3q, 11q, and 18q were more prevalent for invasive ductal carcinoma than for invasive lobular carcinoma (Aldaz et al., 1995). However, 8p losses or imbalances were observed in 36% of invasive lobular tumors but only 14% of invasive ductal carcinomas. Interestingly, microsatellite instability was observed in almost 40% of lobular carcinomas, but only 13% of ductal carcinomas (Aldaz et al., 1995). This phenomenon of microsatellite instability, also known as RER+ phenotype, is identified by allele size differences between tumor and matching normal controls. First described as a characteristic of tumors from patients carrying an autosomal dominant predisposition to tumors of the colon and endometrium, microsatellite instability has been linked to defects in a group of human mismatch repair genes: *hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2* (Altonen et al., 1993; Fischel et al., 1993; Bronner et al., 1994). Resultant errors in DNA repair are believed to be the cause of the observed genomic instability phenomenon. These data suggest that invasive lobular carcinomas may arise by a mechanism of carcinogenesis different from that of ductal breast carcinomas and appear to constitute a distinct pathologic entity.

### Targets of Allelic Loss

Chromosome arm 17p, as previously discussed, is subject to allelic loss in more than 50% of invasive ductal carcinomas, and approximately 30% of noninvasive ductal carcinomas (Radford et al., 1993; Aldaz et al., 1995; Radford et al., 1995). This high frequency of allelic loss suggests that a tumor suppressor of relevance to breast tumorigenesis resides in this region. Indeed, tumor suppressor *p53* is located in this chromosome arm and is known to harbor somatic mutation in 25 to 45% of primary breast carcinomas (Osborne et al., 1991). In addition, germline *p53* mutations have been detected and shown to be causative in families with Li-Fraumeni cancer predisposition syndrome (Malkin et al., 1990; Srivastava et al., 1990). Breast cancer is one of the neoplasms affecting patients with this syndrome. In tumors from patients with Li-Fraumeni syndrome, loss of the wild-type allele is observed with retention of the mutant *p53* allele. Functional studies of cells with mutant *p53* indicate a change of phenotypes, including cellular immortalization, loss of growth suppression, and fourfold increase in protein half-life which leads to *p53* accumulation. Accumulation of *p53* protein, observed by immunohistochemical analysis in roughly 30-50% of sporadic breast carcinomas, was proposed to be an indicator of higher risk of recurrence in patients with tumors positive for *p53* expression (reviewed by Ozbun and Butel, 1995). It is possible that early in breast tumor development, *p53* inactivation through mutation and LOH may be intrinsically linked to the development of subsequent further genomic instability as suggested by findings in experimental model of carcinogenesis (Donehower et al., 1995).

Although *p53* is the most likely candidate for allelic loss on 17p, other reports indicate that there may exist another distinct locus that may be a target of allelic loss. In an analysis of 141 breast tumors, Cornelis et al. observed a strong association between *p53* mutation and allelic loss of the *p53* locus (Cornelis et al., 1994). However, in cases where *p53* mutation was not observed, allelic loss of distal region 17p13.3 was always observed, sometimes without *p53* allele loss. Similar findings of distal deletion of 17p were also observed in DCIS (Radford et al., 1995). While these findings support the existence of a

second gene as target of allelic loss, further studies are needed to address this issue.

The long arm of chromosome 17, also frequently affected by allelic imbalance in both familial and sporadic breast cancers, has recently been subjected to extensive analysis because 17q has been linked to familial breast cancer (Hall et al., 1990). As a result, the *BRCA1* gene was isolated by positional cloning and mutations found to cosegregate with the predisposing haplotype in affected kindreds (Miki et al., 1994). However, when sporadic breast tumors with allelic loss of 17q were examined for *BRCA1* coding sequence alterations, only about 10% of those with LOH revealed any change of sequence, and those mutations were found to be germinal (Futreal et al., 1994). Cellular mislocalization of the *BRCA1* protein has since been reported in sporadic breast tumors, although other groups have not been able to confirm these results (Chen et al., 1995). It remains to be determined what role, if any, *BRCA1* plays in sporadic breast cancer. Another known suppressive gene localized in this region, *nm23* or *NME1*, has been shown to undergo allelic loss in as much as 60% of breast carcinomas (Leone et al., 1991). However, analysis of *NME1* has not revealed evidence of mutations (Cropp et al., 1994). An additional possible explanation for allele loss is the existence of a yet-unidentified gene within this region as the target of allelic loss.

Loss of the *RB1* region 13q14 has been reported for numerous neoplasms including small cell lung carcinoma, bladder carcinoma, osteosarcoma, and breast carcinoma (reviewed by Cox, Chen and Lee, 1994). These losses appear to be relatively early losses in some tumors since 15-20% of tumors at the DCIS stage reveal allelic loss of 13q (Aldaz et al., 1995; Radford et al., 1995). However, when allelic loss and expression are examined in the same breast tumors, no correlation between the two is observed, suggesting that Rb inactivation is not acquired by allelic loss and that another gene may be the target of such inactivation (Borg et al., 1992). More recently, linkage analysis of high-risk breast cancer families localized a second breast cancer susceptibility locus, *BRCA2*, to chromosome 13q12-13 (Wooster et al., 1994). This suggested that the *BRCA2* gene may be involved in sporadic breast cancer as well. However, similar to the findings with *BRCA1* on 17q, when sporadic breast tumors were analyzed for mutation of *BRCA2*, mutations were infrequent, indicating that *BRCA2* is not the gene being targeted by loss (Miki et al., 1996; Teng et al., 1996; Lancaster et al., 1996). Brush-1 is another gene that has been mapped to 13q12-13, proximal to *RB1*. Analysis of Brush-1 expression indicated it to be low to absent in 6 of 13 breast cancer lines and decreased in four of four tumors showing LOH of 13q12-13 (Schott et al., 1994). However, no sequence analysis has yet been reported, and the question of whether decreased expression of Brush-1 results from allelic loss involving large regions of another gene has yet to be addressed.

Chromosomal region 9p21, as previously discussed, has been shown to be affected by allelic loss in 58% of invasive ductal carcinomas and 30% of DCIS, suggesting it may be involved in breast tumorigenesis (Brenner and Aldaz, 1995; Fujii et al., 1996). Previously, the *p16<sup>INK4a</sup>/MTS1/CDKN2* tumor suppressor gene has been identified within this region by positional cloning and shown to be affected in 60% of breast carcinoma lines (Kamb et al., 1994). However, when primary breast tumors were analyzed in our laboratory for mutation of the *CDKN2* coding region, few mutations were found (Brenner and Aldaz, 1995). More recent analysis, including FISH determination of gene copy number, methylation of the 5' region, and analysis of expression, indicate that *p16* is indeed affected in 40 - 60% of breast tumors (Brenner et al., 1996). This observation of inactivation substantiates a role

for *p16* inactivation in the tumorigenesis of the breast and as a target of 9p allelic loss.

Chromosome 16q has been suggested as a site for the occurrence of primary cytogenetic structural abnormalities in the development of breast cancer (Dutrillaux, Gerbault-Seureau, and Zafrani, 1990; Pandis et al., 1992). In particular the long arm of chromosome 16 was shown to systematically participate in nonrandom translocations with chromosome 1 and 16q deletions were also frequently observed. Breast cancer allelotypic studies have also systematically shown the common occurrence of allelic losses affecting the long arm of chromosome 16 (Sato et al., 1990; Tsuda et al., 1994; Cleton-Jansen et al., 1994). In addition to our observations (Aldaz et al., 1995), other investigators have also reported the occurrence of frequent allelic losses affecting chromosome 16q in DCIS (Tsuda et al., 1994; Radford et al., 1995).

It has been suggested that probably more than one putative tumor suppressor locus of interest in breast cancer resides in 16q. At least two regions of chromosome 16q have consistently been previously reported to show LOH: 16q21 and 16q24.2-qter (Tsuda et al., 1994; Cleton-Jansen et al., 1994; Sato et al., 1990). Very recently, by performing a high-resolution allelotyping of chromosome 16 in DCIS lesions, we have identified three distinct regions with a very high incidence (about 70% or more) of allelic losses among informative DCIS samples (Chen et al., 1996). Two of the regions agree with previously described areas: 16q21 at locus D16S400 and 16q24.2 at locus D16S402. However, the region with the highest incidence of LOH observed in our study lies between markers D16S515 and D16S516 (Figure 1). Within this region the D16S518 locus was the most frequently affected: 20 of 26 DCIS tumors (77%) showed LOH at this locus. These observations strongly suggest that a putative tumor suppressor gene(s) may possibly be harbored at or in the vicinity of this locus. On the basis of a YAC contig spanning the region of interest, we can estimate that the minimum region with the highest frequency of LOH is no larger than 2-3 Mb. (Chen et al., 1996). Furthermore, on the basis of the cytogenetic location of markers D16S504 and D16S516 and the distance to D16S518, this area should be contained within bands 16q23.3-q24.1. This region appears different from another area of frequent LOH more distally located at locus D16S402 in band 16q24.2. Both areas are 17 cM apart according to the Genethon Linkage Map (March 1996) and several megabases away according to a comprehensive chromosome 16 physical and genetic map. Further studies are necessary to identify the target gene(s).

It will be particularly important to analyze for the occurrence of allelic losses at the mentioned chromosome 16 regions in other less advanced hyperplastic breast lesions. This analysis will be useful in our understanding of breast carcinogenesis and may help in the identification of markers with diagnostic or prognostic significance.

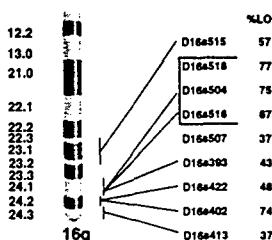


Figure 1. Chromosome arm 16q allelic loss in preinvasive breast carcinomas.

## CELL CYCLE IN BREAST CANCER

Normal cell division in eukaryotes proceeds through an orderly cascade of events manifested as a cell cycle. The machinery responsible for such progress includes a hierarchy of proteins and complexes each exerting an effect on the next. At the top of this hierarchy are the cyclin subunits, whose expression and stability oscillate in a phase-dependent manner. Further, the expression of certain cyclin genes can be upregulated by different mitogenic stimuli, for example, the upregulation of cyclin D1 by estrogen (Altucci et al., 1996). Each of these cyclins can associate in a non-promiscuous manner with specific cyclin-dependent kinases (CDKs). When bound, the cyclins result in activation of CDK activity. However, these cyclins are in competition with CDK inhibitors, which have the ability to displace the cyclin and form an inactive complex with the CDKs. When CDKs are active, they phosphorylate, and hence inactivate, other proteins with transcription-repressing activity (Reviewed by Sherr et al., 1994).

Of the restriction points, G1 to S is best characterized in breast cancer. The players involved in this restriction point include cyclins D1-D3; CDKs 4 and 6, the inhibitors of those CDKs *p15*, *p16*, and *p18*; and the substrate of the CDKs, the *Rb* protein (Figure 2). Collectively, these proteins are known elements responsible for regulation of progression through G1, and loss of function or disregulation of expression of an individual protein can lead to loss of cell cycle regulation and proliferation. Of these proteins, the *Rb* protein, cyclin D1, and *p16* have all been observed to be affected in breast tumorigenesis. As previously mentioned, cyclin D1 has been shown to be both amplified in 10-20% of breast tumors and overexpressed in the majority of breast tumors (Gillet et al., 1994; Bartkova et al., 1994; Weinstat-Saslow et al., 1995). When cyclin D1 is over-abundant, it competes with *p16* for heterodimerization with the CDKs; when cyclin D1 is bound to a CDK, it positively regulates the activity of the CDK which is able to phosphorylate and inactivate *Rb*. Inactivation of *Rb* itself has been described in breast cancer, and when multiple modes of inactivation are accounted for, *Rb* is inactivated in approximately 20% of breast cancers (Borg et al., 1992; Varley et al., 1989). In addition, analyses of *Rb* and *p16* have shown an inverse relationship in expression of the two genes in the vast majority of tumor lines studied (Okamoto et al., 1994; Parry et al., 1995). This is true in breast tumor cell lines as well: in those lines retaining *Rb* expression, *p16* is deleted, mutated, or otherwise affected, and its expression is often undetectable. In contrast, those cell lines retaining *p16* expression often lack expression of *Rb*. Further, when primary breast tumors are analyzed for *p16* expression, approximately 50% show loss of expression due to homozygous deletion, methylation of the 5' region, and rarely by mutation (Brenner et al., 1996). While *CDK4* has not been extensively studied in breast cancer, other neoplasms show overexpression or mutation of the *p16* binding site (He et al., 1994; Zuo et al., 1996). Thus, it appears that mutation or disruption of either *Rb* or *p16* expression or overexpression of cyclin D1 or possibly *CDK4* is sufficient to eliminate this pathway's control of cell cycle progression. The high cumulative rate of alterations affecting these proteins in breast cancer suggests that abrogation of the G1 restriction point may be necessary for breast tumorigenesis.

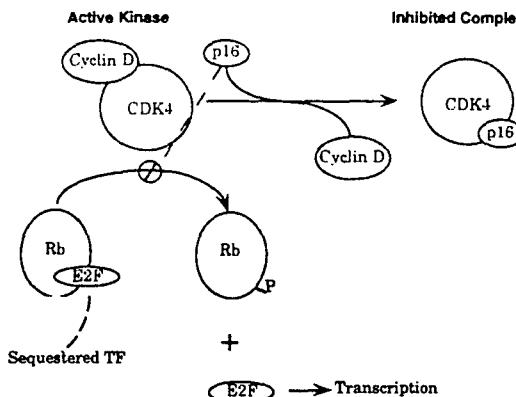


Figure 2. Schematic representation of G1/S restriction point interactions.

The CDK inhibitor *p21*<sup>WAF1/CIP1</sup> is known to be another negative regulator of the cell cycle. Unlike *p16*, it is known to be a universal inhibitor of CDKs thereby inducing arrest at both the G1/S and G2/M restriction points (Xiong et al., 1993). Further, *p21* also complexes with proliferating cell nuclear antigen *in vitro*, resulting in inhibition of DNA replication (Waga et al., 1994). Because *p21* gene transcription is regulated by *p53*, it has been suggested that *p53*-dependent cell cycle arrest is mediated by *p21*. Indeed, *p21* nullizygous mice fibroblasts fail to undergo G1 arrest following DNA damage although apoptosis is unaffected in these same cells (Brugarolas et al., 1995). As previously mentioned, positive *p53* gene detection has been shown in as much as half of breast carcinomas, and *p53* has been shown to be associated with *p53* mutation and a higher risk of recurrence (reviewed by Ozbun and Butel, 1995). This would suggest that an additional possible consequence of *p53* inactivation in the tumorigenesis of the breast is the abrogation of cell cycle arrest through loss of transcriptional activation of *p21*.

## SUMMARY

Breast cancer is a complex disease in which numerous genetic aberrations occur. It is unclear which, if any, of these abnormalities are causative of breast tumorigenesis. However, on the basis of the currently accepted view of breast cancer as a multistep process, it is possible that specific abnormalities may be required in the progression from a normal breast epithelial cell to an invasive tumor cell. Figure 3 shows a schematic putative model of breast cancer progression based primarily on epidemiological and histopathological studies (Page and DuPont, 1992). Advances in methodology have allowed us to more precisely determine the approximate chronology of some of these aberrations and the possible roles each plays in the formation of malignancy. Simplistically, one could speculate that it is the early loss of cell cycle control in the presence of a mitogenic stimulus that allows a cell to divide unchecked. Such uncontrolled proliferation in the absence of wild type *p53* would yield a high level of genomic instability. As proliferation continues, numerous additional

chromosomal abnormalities occur, and increased tumor heterogeneity would be observed as distinct subpopulations emerge in the evolution toward a progressively more aggressive phenotype. However, much still remains to be learned to gain a full understanding of the key players behind the genetic evolution of breast cancer. Only by analyzing preinvasive and putative early stages of breast cancer will we be able to characterize the most probable sequence of genomic abnormalities.

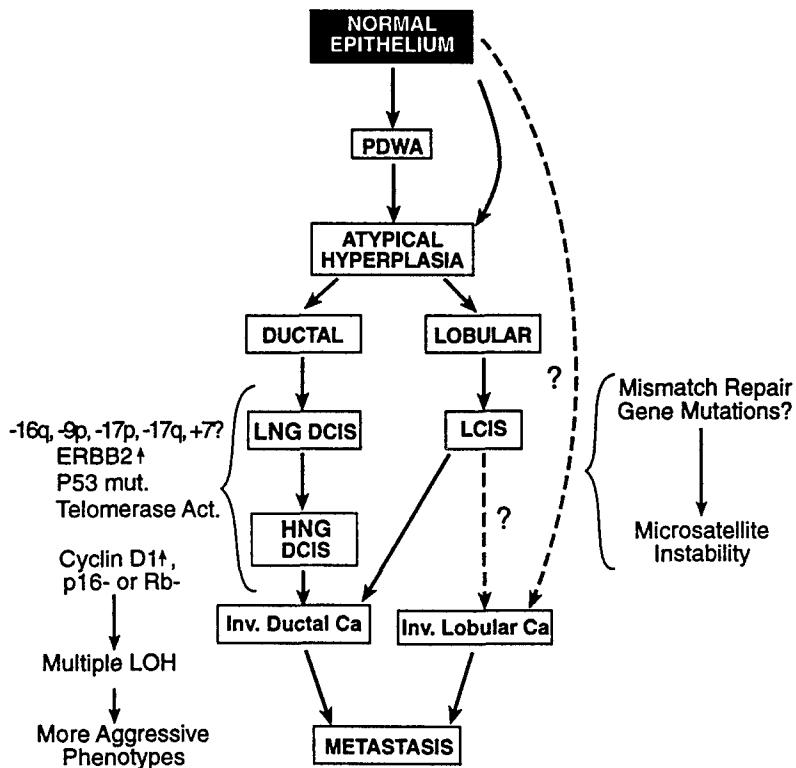


Figure 3. Schematic putative model of breast cancer progression.

## ACKNOWLEDGEMENTS

The authors wish to thank contributing colleagues Taiping Chen, Andzrej Bednarek, Abhaya Paladugu, Hui Wang, and Qiao Ying Liao, and Michelle Gardiner for her secretarial assistance. This work was supported by Grants DAMD 17-94-J-4078 and DAMD 17-96-1-6252 from the U.S. Army Breast Cancer Program and NIH Grant R01 CA59967.

## REFERENCES

Altonen LA, Peltomaki P, Leach FS, Sistonen P, Pylkkanen L, Mecklin JP, Jarvinen H, Powell SM, Jen J, Hamilton SR, et al (1993): Clues to the pathogenesis of familial colorectal cancer. *Science* 260:812-816.

Aldaz CM, Chen T, Sahin A, Cunningham J, Bondy M (1995): Comparative allelotype of *in situ* and invasive human breast cancer: High frequency of microsatellite instability in lobular breast carcinomas. *Cancer Res* 55:3976-3981.

Altucci L, Addeo R, Cicatiello L, et al (1996): 17 $\beta$ -estradiol induces cyclin D1 gene transcription, p36D1-p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells. *Oncogene* 12:2315-2324.

Bartkova J, Lukas J, Muller H, Lutzht D, Strauss M, Bartek J (1994): Cyclin D1 protein expression and function in human breast cancer. *Intl J Cancer* 57:353-361.

Berger MS, Locher GW, Saurer S, Gullick WJ, Waterfield MD, Groner B, Hynes NE (1988): Correlation of C-ERBB-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. *Cancer Res* 48:1238-1243.

Borg A, Zhang Q-X, Alm P, Olsson H, Sellberg G (1992): The retinoblastoma gene in breast cancer: allele loss is not correlated with loss of gene protein expression. *Cancer Res* 52:2991-2994.

Boring CC (1994): Cancer statistics. *CA Cancer J Clin* 44:7-26.

Bouchard L, Lamarre L, Tremblay PJ, Jolicoeur P (1989): Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/C-NEU oncogene. *Cell* 57:931-936.

Brenner AJ, Aldaz CM (1995): Chromosome 9p allelic loss and *p16/CDKN2* in breast cancer and evidence of *p16* inactivation in immortal breast epithelial cells. *Cancer Res* 55:2892-2895.

Brenner AJ, Paladugu A, Wang H, Olopade OI, Dreyling MH, Aldaz CM (1996): Loss of

p16 is preferential over alternative p19 in breast cancer. *Clinical Cancer Res (in press)*.

Bronner CE, Baker SM, Morrison PT, Warren G, Smith LG, Lescoe MK, Kane M, Earabinc C, Lipford J, Lindblom A, Tannergard P, Bollag RJ, Godwin AR, Ward DC, Nordenskjold M, Fishel R, Kolodner R, Liskay RM (1994): Mutation in the DNA mismatch repair gene homologue *hMLH1* is associated with hereditary non-polyposis colon cancer. *Nature* 368:258-261.

Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T, Hannon GJ (1995): Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature*. 377(6549):552-7.

Chen T, Sahin A, and Aldaz CM (1996): Deletion Map of Chromosome 16q in Ductal Carcinoma *In Situ* of the Breast: Refining a Putative Tumor Suppressor Gene Region. (Submitted for publication).

Chen Y, Chen CF, Riley DJ, Allred DC, Chen PL, Von Hoff D, Osborne CK, Lee WH (1995): Aberrant subcellular localization of BRCA1 in breast cancer. *Science* 270:789-791.

Cleton-Jansen AM, Moerland EW, Kuipers-Dijkshoorn NJ, Callen DF, Sutherland GR, Hansen B, Devilee P, Cornelisse CJ (1994): At least two different regions are involved in allelic imbalance on chromosome arm 16q in breast cancer. *Genes, Chromos. & Cancer*, 9:101-107.

Comings DE (1973): A general theory of carcinogenesis. *Proc Natl Acad Sci USA* 70:3324-3328.

Cornelis RS, van Vliet M, Vos CBJ, Cleton-Jansen A-M, van de Vijver MJ, Peterse JL, Khan PM, Borresen A-L, Cornelisse CJ, Devilee P (1994): Evidence for a gene on 17p13.3, distal to TP53, as a target for allele loss in breast tumors without *p53* mutations. *Cancer Res* 54:4200-4206.

Coussens L, Yang-Feng TL, Liao YC, Chen E, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke U, et al (1985): Tryosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with Neu oncogene. *Science* 230:1132-1139.

Cropp CS, Lidereau R, Leone A, Liscia D, Cappa APM, Campbell G, Barker E, Le Doussal V, Steeg PS, Callahan R (1994): NME1 protein expression and loss of heterozygosity mutations in primary human breast tumors. *J. Natl. Cancer Inst.* 86:1167-1169.

Cox LA, Chen G, Lee E Y-H P (1994): Tumor suppressor genes and their roles in breast cancer. *Breast Cancer Res Treat* 32:19-38.

Devilee P, Cornelisse CJ (1994): Somatic genetic changes in human breast cancer. *Biochimica*

et Biophysica Acta 1198:113-130.

Dib C, Fauré S, Fizames C, Samson D, Drouot N, Vignal A, Millasseau P, Marc S, Hazan J, Seboun E, Lathrop M, Gyapay G, Morissette J, Weissenbach J (1996): A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature* 380:152-154.

Dickson C, Fantl V, Gillett C, Brookes S, Bartek J, Smith R, Fisher C, Barnes D, Peters G (1995): Amplification of chromosome band 11q13 and a role for cyclin D1 in human breast cancer. *Cancer Let* 90:43-50.

Donehower LA, Godley LA, Aldaz CM, Pyle R, Shi YP, Pinkel D, Gray J, Bradley A, Medina D, Varmus HE (1995): Deficiency of p53 accelerates mammary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability. *Genes & Development* 9(7):882-95.

Dutrillaux B, Gerbault-Seureau M, Zafrani B (1990): Characterization of chromosomal anomalies in human breast cancer. A comparison of 30 paradigm cases with few chromosome changes. *Cancer Genet Cytogenet* 49:203-217.

Easton DF, Bishop DT, Ford D, Crockford GP (1993): Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. The Breast Cancer Linkage Consortium. *Am J Hum Gene* 52:678-701.

Feuer EJ (1993): The lifetime risk of developing breast cancer. *J Natl Cancer Inst* 85:892-897.

Fishel R, Lescoe MK, Rao MRS, Copeland NG, Jenkins NA, Garber J, Kane M, Kolodner R (1993): The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis colon cancer. *Cell* 75:1027-1038.

Fujii H, Marsh C, Cairns P, Sidransky D, Gabrielson E (1996): Genetic divergence in the clonal evolution of breast cancer. *Cancer Res* 56:1493-1497.

Futreal PA, Liu Q, Shattuck-Eidens D, Cochran C, Harshman K, Tavtigian S, Bennett LM, Haugen-Strano A, Swensen J, Miki Y, Eddington K, McClure M, Frye C, et al (1994): *BRCA1* mutations in primary breast and ovarian carcinomas. *Science* 266:120-122.

Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, Barnes D, Peters G (1994): Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res* 54:1812-1817.

Goodrich DW, Lee W-H (1993): Molecular characterization of the retinoblastoma susceptibility gene. *Biochimica et Biophysica Acta* 1155:43-61.

Gray JW, Collins C, Henderson IC, Isola J, Kallioniemi A, Kallioniemi O-P, Nakamura H,

Pinkel D, Stokke T, Tanner M, Waldman F (1994): Molecular Cytogenetics of Human Breast Cancer. 645-652.

Hall JM, Lee MK, Newman B, Morrow JE, Anderson LA, Huey B, King MC. (1990): Linkage of early-onset familial breast cancer to chromosome 17q21. *Science* 250:1684-1689.

Harris H, Miller OJ, Klein G, Worst P, Tachibana T (1969): Suppression of malignancy by cell fusion. *Nature* 223:363-368.

He J, Allen JR, Collins VP, Allalunis-Turner MJ, Godbout R, Day III RS, James CD (1994): CDK4 amplification is an alternative mechanism to *p16* gene homozygous deletion in glioma cell lines. *Cancer Res* 54:5804-5807.

Isola JJ, Kallioniemi OP, Chu LW, Fuqua SA, Hilsenbeck SG, Osborne CK, Waldman FM (1995): Genetic aberrations detected by comparative genomic hybridization predict outcome in node-negative breast cancer. *Am J Path* 147:905-911.

Jardines L, Weiss M, Fowble B, Greene M (1993): *neu(c-erbB-2/HER2)* and the epidermal growth factor receptor (EGFR) in breast cancer. *Pathobiology* 61:268-282.

Kallioniemi A, Kallioniemi O-P, Piper J, Tanner M, Stokke T, Chen L, Smith HS, Pinkel D, Gray JW, Waldman FM (1994): Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. *Proc Natl Acad Sci USA* 91:2156-2160.

Kallioniemi A, Kallioniemi O-P, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D (1992a): Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* 258:818-820.

Kallioniemi OP, Kallioniemi A, Kurisu W, Thor A, Chen LC, Smith HS, Waldman FM, Pinkel D, Gray JW (1992b): ERBB2 amplification in breast cancer analyzed by fluorescence in situ hybridization. *Proc Natl Acad Sci USA* 89:5321-5325.

Kamb A, Gruis N, Weaver-Feldhaus J, Qingyun L, Harshman K, Tavtigian S, Stockert E, Day R, Johnson B, Skolnick M (1994): A cell cycle regulator potentially involved in genesis of many tumor types. *Science* 264:436-440.

Klein G, Bregula U, Wiener F (1971): The analysis of malignancy by cell fusion. *J Cell Sci* 8:659-672.

Knudson AG (1971): Mutation and Cancer: Statistical study of retinoblastoma. *Proc Natl Acad Sci USA* 68:820-823.

Lammie GA, Peters G (1991): Chromosome 11q13 abnormalities in human cancer. *Cancer*

Cells 3:413-420.

Lancaster JM, Wooster R, Mangion J, et al (1996): BRCA2 mutations in primary breast and ovarian cancers. *Nature Genetics* 13:238-240.

Leone A, McBride OW, Weston A, Wang MG, Anglard P, Cropp CS, Goepel JR, Lidereau R, Callahan R, Linehan WM, Rees RC, Harris CC, Liotta LA, and Steeg PS (1991): Somatic allelic deletion of *nm23* in human cancer. *Cancer Res* 51:2490-2493.

Litt M, Luty JA (1989): A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am J Hum Genet* 44:397-401.

Malkin D, Li FP, Strong LC, Fraumeni JF Jr, Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA, et al (1990): Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* 250:1233-1238.

Miki Y, Katagiri T, Kasumi F, Yoshimoto T, Nakamura Y (1996): Mutation analysis in the BRCA2 gene in primary breast cancers. *Nature Genetics* 13:245-247.

Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, et al (1994): A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. *Science* 266:66-71.

Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P (1988): Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell* 54:105-115.

Okamoto A, Demetrick DJ, Spillare EA, Hagiwara K, Hussain SP, Bennett WP, Forrester K, Gerwin B, Serrano M, Beach DH, Harris CC (1994): Mutations and altered expression of p16<sup>INK4</sup> in human cancer. *Proc Natl Acad Sci* 91:11045-11049.

Osborne RJ, Merlo GR, Mitsudomi T, Venesio T, Liscia DS, Cappa APM, Chiba I, Takahashi T, Nau MM, Callahan R, Minna JD (1991): Mutations in the *p53* gene in primary human breast cancers. *Cancer Res* 51:6194-6198.

Ozbun MA, Butel JS (1995): Tumor suppressor p53 mutations and breast cancer: a critical analysis. *Adv Cancer Res* 66:71-141.

Page DL, Dupont WD (1992): Benign breast disease: indicators of increased breast cancer risk. *Cancer Detection & Prevention* 16:93-97.

Pandis N, Heim S, Bardi G, et al (1992): Whole-arm t(1;16) and i(1q) as sole anomalies identify gain of 1q as a primary chromosomal abnormality in breast cancer. *Genes Chromosomes & Cancer*, 5:235-238.

Parry D, Bates S, Mann DJ, Peters G (1995): Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of p16<sup>INK4/MTS1</sup> tumour suppressor product. *EMBO J* 14:503-511.

Radford DM, Fair KL, Phillips NJ, Ritter JH, Steinbrueck T, Holt MS, Donis-Keller H (1995): Allelotyping of ductal carcinoma *in situ* of the breast: deletion of loci on 8p, 13q, 16q, 17p and 17q. *Cancer Res* 55:3399-3405.

Radford DM, Fair K, Thompson AM, Ritter JH, Holt M, Steinbrueck T, Wallace M, Wells SA Jr, Donis-Keller HR (1993): Allelic loss on a chromosome 17 in ductal carcinoma *in situ* of the breast. *Cancer Res* 53:2947-2949.

Ravdin PM, Chamness GC (1995): The *c-erbB-2* proto-oncogene as a prognostic and predictive marker in breast cancer: a paradigm for the development of other macromolecular markers - a review. *Gene* 159:19-27.

Ryan KM, Birnie GD (1996): Myc oncogenes: the enigmatic family. *Biochem J* 314:713-721.

Sato T, Tanigami A, Yamakawa D, et al (1990): Allelotyping of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. *Cancer Res.*, 50:7184-7189.

Schott DR, Chang JN, Deng G, Kurisu W, Kuo WL, Gray J, Smith HS (1994): A candidate tumor suppressor gene in human breast cancers. *Cancer Res* 54:1393-1396.

Sherr CJ (1994): G1 phase progression: cycling on cue. *Cell* 79:551-555.

Shiu RPC, Watson PH, Dubik D (1993): *c-myc* oncogene expression in estrogen-dependent and -independent breast cancer. *Clin Chem* 39:353-355.

Sicinski P, Donaher JL, Parker SB, Li T, Fazeli A, Gardner H, Haslam SZ, Bronson RT, Elledge SJ, Weinberg RA (1995): Cyclin D1 provides a link between development and oncogenes in the retina and breast. *Cell* 82:621-630.

Silverstein MJ, Lewinsky BS, Waisman JR, Gierson ED, Colburn WJ, Senofsky GM, Gamagami P (1994): Infiltrating lobular carcinoma. Is it different from infiltrating duct carcinoma? *Cancer* 73:1673-1677.

Silverstein MJ, Lewinsky BS, Waisman JR, Gierson ED, Colburn WJ, Senofsky GM, Gamagami P (1994): Infiltrating lobular carcinoma. It is different from infiltrating duct carcinoma? *Cancer* 73:1673-1677.

Srivastava S, Zou ZQ, Pirolo K, Blattner W, Chang EH (1990): Germ-line transmission of

a mutated p53 gene in a cancer-prone family with Li-fraumeni syndrome. *Nature* 348:747-749.

Tavassoli FA (1992): *Pathology of the Breast*, Norwalk, CT: Appleton & Lange.

Tavtigian SV, Simard J, Rommens J, et al (1996): The complete BRCA2 gene and mutations in chromosome 13q-linked kindreds. *Nature Genetics* 12:333-337.

Teng DH, Bogden R, Mitchell J, et al (1996): Low incidence of BRCA2 mutations in breast carcinoma and other cancers. *Nature Genetics* 13:241-244.

Thompson F, Emerson J, Dalton W, Yang J-M, McGee D, Villar H, Knox S, Massey K, Weinstein R, Bhattacharyya A, Trent J (1993): Clonal chromosome abnormalities in human breast carcinomas I. Twenty-eight cases with primary disease. *Genes, Chromo Cancer* 7:185-193.

Trent J, Yang J-M, Emerson J, Dalton W, McGee D, Massey K, Thompson F, Villar H (1993): Clonal chromosome abnormalities in human breast carcinomas II. Thirty-four cases with metastatic disease. *Genes, Chromo Cancer* 7:194-203.

Tsuda H., Callen DF, Fukutomi T., Nakamura Y, Hirohashi S (1994): Allele loss on chromosome 16q24.2-qter occurs frequently in breast cancer irrespectively of differences in phenotype and extent of spread. *Cancer Tes.*, 54:513-517.

Varley JM, Armour J, Swallow JE, Jeffreys AJ, Ponder BA, T'Ang A, Fung YK, Brammar WJ, Walker RA (1989): The retinoblastoma gene is frequently altered leading to loss of expression in primary breast tumours. *Oncogene* 4:725-729.

Varley JM, Swallow JE, Brammar WJ, Whittaker JL, Walker RA (1987): Alterations to either C-ERBB-2 (NEU) or C-MYC proto-oncogenes in breast carcinomas correlate with poor short-term prognosis. *Oncogene* 1:423-430.

Waga S, Hannon GJ, Beach D, Stillman B (1994): The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature*. 369:574-578.

Wang TC, Cardiff RD, Zukerberg L, Lees E, Arnold A, Schmidt EV (1994): Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature* 369:669-671.

Weber JL, May PE (1989): Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am J Hum Genet* 44:388-396.

Weinstat-Saslow D, Merino MJ, Manrow RE, Lawrence JA, Bluth RF, Wittenbel KD, Simpson JF, Page DL, Steeg PS (1995): Overexpression of cyclin D mRNA distinguishes invasive and in situ breast carcinomas from non-malignant lesions. *Nature Med* 1:1257-

1260.

Wooster R, Neuhausen SL, Mangion J, Quirk Y, Ford D, Collins N, Nguyen K, Seal S, Tran T, Averill D, Fields P, Marshall G, Narod S, et al (1994): Localization of a breast cancer susceptibility gene, *BRCA2*, to chromosome 13q12-13. *Science* 265:2088-2090.

Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N, et al (1995): Identification of the breast cancer susceptibility gene *BRCA2*. *Nature* 378:789-792.

Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D (1993): p21 is a universal inhibitor of cyclin kinases. *Nature* 366(6456):701-704.

Yeager T, Stadler W, Belair C, Puthenveettil J, Olopade O, Reznikoff C (1995): Increased p16 levels correlate with pRb alterations in human urothelial cells. *Cancer Res* 55:493-497.

Zhou D, Battifora H, Yokota J, Yamamoto T, Cline MJ (1987): Association of multiple copies of the c-erbB-2 oncogene with spread of breast cancer. *Cancer Res* 47:6123-6125.

Zuo L, Weger J, Yang Q, Goldstein AM, Tucker MA, Walker GJ, Hayward N, Dracopoli NC (1996): Germline mutations in the *p16INK4a* binding domain of CDK4 in familial melanoma. *Nature Genetics* 12:97-99.

**NCBI Entrez**      **Nucleotide QUERY**      **BLAST** **Entrez** **?**

Other Formats:

**FASTA****Graphic**

**LOCUS** AF179633 96371 bp DNA **PRI** 05-SEP-1999  
**DEFINITION** Homo sapiens chromosome 16 map 16q23.3-q24.1 sequence.  
**ACCESSION** AF179633  
**NID** g5823550  
**VERSION** AF179633.1 GI:5823550  
**KEYWORDS**  
**SOURCE** human.  
**ORGANISM** Homo sapiens  
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia;  
Eutheria; Primates; Catarrhini; Hominidae; Homo.  
**REFERENCE** 1 (bases 1 to 96371)  
**AUTHORS** Bednarek,A.K., Chen,T., Laflin,K.J., Hawkins,K.A., Liao,Q. and Aldaz,C.M.  
**TITLE** Direct Submission  
**JOURNAL** Submitted (23-AUG-1999) Carcinogenesis, University of Texas, M.D. Anderson Cancer Center, Science Park-Research Division, Park Road 1C, P.O. Box 389, Smithville, TX 78957, USA  
**FEATURES**  
**source** Location/Qualifiers  
1..96371  
/organism="Homo sapiens"  
/db\_xref="taxon:9606"  
/chromosome="16"  
/map="16q23.3-q24.1; between D16S518 and D16S516"  
/clone="BAC249B4; BAC286F3; BAC112B17; BAC36022"  
/clone\_lib="Research Genetics, Inc. CITB-HSP-C library"  
repeat region complement(265..541)  
/rpt\_family="Alu"  
/rpt\_type=dispersed  
1099..1397  
/rpt\_family="Alu"  
/rpt\_type=dispersed  
repeat region complement(2501..2778)  
/rpt\_family="Alu"  
/rpt\_type=dispersed  
repeat region complement(3376..3606)  
/rpt\_family="Alu"  
/rpt\_type=dispersed  
repeat region complement(3729..3812)  
/rpt\_family="MIR"  
/rpt\_type=dispersed  
STS 7553..7684  
/db\_xref="dbSTS:G22903"  
repeat region complement(8121..8403)  
/rpt\_family="Alu"  
/rpt\_type=dispersed  
repeat region complement(8601..8864)  
/rpt\_family="Alu"  
/rpt\_type=dispersed  
repeat region 9337..9590  
/rpt\_family="Alu"  
/rpt\_type=dispersed  
repeat region complement(10106..10193)  
/rpt\_family="MIR"  
/rpt\_type=dispersed  
repeat region 10503..10750  
/rpt\_family="Alu"  
/rpt\_type=dispersed  
repeat region 11491..11787  
/rpt\_family="Alu"  
/rpt\_type=dispersed  
repeat region 14683..14864  
/rpt\_family="Alu"  
/rpt\_type=dispersed  
repeat region complement(16507..16781)  
/rpt\_family="Alu"  
/rpt\_type=dispersed  
repeat region 17333..17625  
/rpt\_family="Alu"

repeat region /rpt\_type=dispersed  
17830..18117 /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
19141..19428 /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
19458..19711 /rpt\_family="MLT2B2"  
repeat region /rpt\_type=dispersed  
21308..21571 /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
23421..23707 /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
complement(25091..25226) /rpt\_family="L1"  
repeat region /rpt\_type=dispersed  
complement(25356..25608) /rpt\_family="MER7"  
repeat region /rpt\_type=dispersed  
complement(25775..26032) /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
27530..27815 /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
28973..29184 /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
complement(29778..29942) /rpt\_family="MLT1"  
repeat region /rpt\_type=dispersed  
complement(32318..32601) /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
complement(32812..33114) /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
34781..34837 /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
35161..35951 /rpt\_family="MER42"  
repeat region /rpt\_type=dispersed  
complement(39543..39810) /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
complement(39994..40187) /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
complement(43566..43874) /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
48563..48668 /rpt\_family="MIR"  
repeat region /rpt\_type=dispersed  
49091..49373 /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
50165..50335 /rpt\_family="MLT1"  
repeat region /rpt\_type=dispersed  
complement(52299..52423) /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
52862..53272 /rpt\_family="MLT2B2"  
repeat region /rpt\_type=dispersed  
complement(54582..54832) /rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
55726..56056 /rpt\_family="MLT1"

repeat region /rpt\_type=dispersed  
complement(56053..56309)  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
57555..57909  
/rpt\_family="THE1"  
repeat region /rpt\_type=dispersed  
58410..58622  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
complement(59901..60181)  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
60238..60516  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
61451..61721  
/rpt\_family="MLT1"  
repeat region /rpt\_type=dispersed  
complement(63697..63934)  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
complement(67062..67361)  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
68170..68305  
/db\_xref="dbSTS:G43876"  
repeat region complement(69661..69955)  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
70746..70866  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
73190..73332  
/db\_xref="dbSTS:G15501"  
repeat region 74482..74622  
/rpt\_family="MER3"  
repeat region /rpt\_type=dispersed  
74482..74535  
/rpt\_family="MER33"  
repeat region /rpt\_type=dispersed  
complement(74584..74626)  
/rpt\_family="MER3"  
repeat region /rpt\_type=dispersed  
76820..77138  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
78678..78951  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
complement(79491..79770)  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
complement(80909..81184)  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
81261..81364  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
complement(84469..84695)  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
85302..85588  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
complement(86554..86724)  
/rpt\_family="MIR"  
repeat region /rpt\_type=dispersed  
complement(88516..88612)  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
89071..89331  
/rpt\_family="Alu"  
repeat region /rpt\_type=dispersed  
complement(90533..90812)

```

/rept_family="Alu"
/rept_type=dispersed
repeat region complement(91380..91658)
/rept_family="Alu"
/rept_type=dispersed
repeat region 91963..92288
/rept_family="Alu"
/rept_type=dispersed
repeat region complement(92304..92499)
/rept_family="MIR"
/rept_type=dispersed
repeat region 93182..93265
/rept_family="MER21"
/rept_type=dispersed
repeat region 93490..94079
/rept_family="MER21"
/rept_type=dispersed
repeat region 94217..94612
/rept_family="L1"
/rept_type=dispersed
repeat region complement(95236..95546)
/rept_family="Alu"
/rept_type=dispersed
repeat region 96212..96360
/rept_family="Alu"
/rept_type=dispersed

```

BASE COUNT 29090 a 20438 c 19962 g 26877 t 4 others  
 ORIGIN

```

1 ctgaaaactga aatgtcatca atccatatac actaaggaga gggacaatgg caggatgggg
61 gtgcacctgc tcggaagagg tatccacaac tcaccactca cttaaaaact ctgtggataa
121 cttcatttcag aaacaaaggt aaggagagaa attgttaatg tgggggtgaga gctaaaaat
181 ttgcccagta agggtttgg ctgcattcaa atgctagaag aataaaatatc ctcattcgaa
241 gctaaaaatag caagggtttgg tttatttttg agatagggtc tagccttgc acccaggtt
301 gagtgcggtg acatgatatac agcttactgc aaccttctacc tcctgggttc aagtgacctt
361 cctgcctcag cctcctgagt agctgggatt atagcacatg ccaccacacc cagctaattt
421 ttgtatTTTT agtagagatg ggatttcacc atgttggcca ggggtgtctc aaactccaa
481 cctcaggggta tctacactgcg ttggcctccc aaagagctgg gattacaggc atgagccacc
541 gtgcctggac tgcgtacttct tctaattcga gaagaaaatg cctatatcca taaaatcact
601 atagaaaacca actgaggcaga agaaccattt tggcgtagac aagccagaaaa gaaaatcatt
661 gatcatttta ttacttcta atgtattaaat ggtgagccaa cctatcatgt acaaataattt
721 tctatctgtt ttaaaccacaa tggagctaa aagaatgtaa tacaaaggca cacagatacc
781 ttacatcaag tcacaattac tcttgcattt aaagcttagac agcaatttgc ttgaggttag
841 agactattcc agcaaggatc ctggcaaaa gttggccctc cgaaatttgc ttgttgcattt
901 ttaatttttctt atgtctaaatg cattggaaac ttttatttctc tgattttcct tggaaaatata
961 ttttggagaa aacctgtata tttagtttctt agaaggctgt gtcgtcaaga aaaaacttaac
1021 taaatttagac taacattttt cagatTTTGC ctgggcagca gtaactacgt agacaaaaatt
1081 ttaccaggcgc caggcacatg ggctcatgcc tgaaccatc gcactttggg aggctgaggc
1141 acgtgaatcg cttaggcctca ggatTTTGA gaccaggctt ggcacacatgg caaaatccca
1201 tctgtacccaa aaaatacaac aacaacaaaa attggcagg catgggtgt tgccctgtg
1261 gtcccagctc ccttgggagg ctgaggttagg aggatcttgc ggcggccagg ggcgggggg
1321 gcagtggcc gtcgtactgc caccacactc cagcctggca acagggcggag actttgtctc
1381 aaaaaaaaaaaa aaaaaaaagaa aaaaaattac cagaagcttt agggggaccga aagatagact
1441 cataatggaa aaaaaaaatgt ctggaaaaac aggagtccagg gtcttataacc tacgtctgac
1501 ctgatctgt atcatakaca agaaggcaga gcattttctt gaatgtgaa ctgatccaa
1561 ctttagaaaca tactccatg ttggggaaac acattttctt tccatctgtt acagtaagt
1621 ctttggaaacc atcttctccc aaaaatgtgtt ttgtcaaaaa atatgtcactc acacttatgt
1681 gaactctcag atggaccgc accaagagta catcagcaag ttccggcaagt gaattccctc
1741 accaataata aataaggaga aaaggaaatg aagatggca ttttggctgg attccaaatg
1801 caaaaacctg cctatctacc ttcaaaaatg tgcaagattt cagtttgcattt aatgttcatc
1861 ttatgtgcattt aattaccatg agtgcatttgc taatttttc ttgttttgcattt gactattaa
1921 acagagaagg aaaggcacta tatactttcg tataccgaat gaggatttgcattt aactcatttt
1981 tgcgtatcga taatcccgagg ggggttttgcattt tacaacacaa aagaaaaaca ggtatgtcaaa
2041 agcagggtgac agtcttaattt ccacagggtac ggggtggaaagc attagagtgtt agtaatggaa
2101 aataaaaaataa aaaaatggctt tttagtgcattt ccatctgtt aaggtgtttt catgttaata
2161 agagagaaaa ctgcaaaatac agcaaaatg tcttcgtttt gtaactgggtt cttttctgtat
2221 gtctgatttgc atccaaacatc tctgaaataa accaccaat agaccccgat tttcttatttgc
2281 acaaataataa gtgtttgaca agttgcatac ggtatcatca actcgatca tgcgtatca tgcgtatca
2341 ttcaactttt tactttatctt accttttgcattt ataaattttt ggtatcatca cggccattaca
2401 ccagatttca aatgttttag aaaaatgtgtt ggctttaactt aaaaatgtgtt accagagacc
2461 ctagagatc cagactgttta ggaatttactt cttcttgcattt tttttttttt ttgagacaga
2521 gtttgcgtt ttttgcgtt gtttgcgtt gtttgcgtt gtttgcgtt gtttgcgtt gtttgcgtt
2581 tgcctccaa gttcaagggc ctctcctggc tcagcttgc accatgtgg gattacaggc
2641 atgtgccacc atgcctggctt aatTTTGTAT tttagtgcattt gacgggggtt cttccatgttt
2701 atcagactgg tctcgaactc cccgacccatg gtgtatccgc tgcctcgcc tcccaaaagcg
2761 ctgggatttgc atggcgatc accatgttgc accaccaat agccaggaaat tccatgttgc tcccaaaat
```



7321 gttgagttgt cctgctgaa atgaaatagt  
7381 attccctttaa gaggccaagt cctcaaaaggc  
7441 ccttcctggc tccaatgcat gtttcacag  
7501 ttgggcctat ctgagggatc attaccact  
7561 ctagtggtaa gtctggccaa aataatactcc  
7621 ttggaacatg tgagtagatg ttctggcaac  
7681 agccaaaaaa aaaaaaaaaa aaaaaaaaaaa  
7741 ggttgcataa tttttgtcca ctacccattg  
7801 aatctgtatg ggagaccatc cagacatctt  
7861 ataccaggcg ctgcagttag ccctggccag  
7921 tttttgaaa tcgaccatg tggaggttagc  
7981 acttgacagg tttagcaact gcgtctgctt  
8041 agatcatgtc tgttcaggat gcagctctct  
8101 ctggcggcataa aatggaaagc tttttttttt  
8161 catgcttagag tgccggccgc ccatctcagc  
8221 cgatttcctt gcctcagcca ctgcagtagc  
8281 gctatTTTTT ttttaataaa ttttagtaga  
8341 ctgacccctt gatccggcca cccggccctc  
8401 ccgtgctcaag ccagaacccctt tcttaactt  
8461 gccactaga atgaagtctt cagagacact  
8521 actcccaggc actaaggcaca tggctggca  
8581 tattttacta ttttattttac ttttgaggc  
8641 agtggtaaaaa taacagctct ctgcagccct  
8701 cagtctccca agtagctggg atgacaggca  
8761 aatattttat agagatgagg tgcgtatgat  
8821 caattaatcc tcccacccca gcctccaaa  
8881 gcctggccctt ccaagttt aaaaatgaatg  
8941 ctgaaacaact tacatttta ccacatctgc  
9001 aaaaaaaaaa aaatcagcat aaggagatag  
9061 ataataggag cttagtcctt cttaattcca  
9121 attcaaaacccctt ctatctaaa caaattttta  
9181 gaaaaggcaca tacataataa gtgcacgact  
9241 accaaagaccg agatggagga agagaaaaag  
9301 ggtgtggtaa cttacaccccg taatctcagt  
9361 tgaggccagg agctccagac cagcctgggc  
9421 acaaagattt gcccaggatg gtgaaatata  
9481 atgggaggac tgcttgagcc ctggaggccca  
9541 cactccagcc tgagcaagag agcggagaccc  
9601 aaaggggccctt ctgcgtatgtt agtaatgtt  
9661 tcggaaggca aaaaaagaga aacgcgggaa  
9721 tgactctggc ctgcaagca atgtgaggta  
9781 tactgaatttccatccatgtg ccctaaggta  
9841 ttgaggctta gagtgattaa tgcaacttgg  
9901 ccacagcccc tttatcccc actgtatattc  
9961 tcctttaact cccttctta aaaaatgt  
10021 gctttaatta taactttgtt gagtcctcag  
10081 cctttagcccc atctttctta tgaaaaaact  
10141 gagcttcatac agagatctca gtaatagcag  
10201 ctatgggttgc cactgttgcatcgtct  
10261 ttataatttc ctgttcttta atttccttatt  
10321 aaacactgggt tagaagggtt gacagaaatt  
10381 ttggcccttgc ttgagttggca tctgagaggg  
10441 ggcatggctg gaagaaaaata ggttgaggg  
10501 ggggtcaca cctgtatattcc cagcgttgg  
10561 caggagttt agaccgcctt ggcacatatg  
10621 aaaaatttagg tgggtatgtt ggtgccttgc  
10681 aggagaattt cttgaacccca gaaggcagag  
10741 ctccagccctg agcaacctgg taatagccat  
10801 cttagtaagcc tcaattttagg atttagattt  
10861 aactggctcc ttggagatct tgcacccct  
10921 gtgcagaagc actggaaact gtgggtggaca  
10981 cagttatcac cctgggttgc tgcaaaacatc  
11041 tcaagttcac ccagtccctc tcctggccact  
11101 gcatacaggat tataagaata aggacgaagg  
11161 cttaggacagc cagcgggggt ttttaccct  
11221 accttcacga aagttggaa ttactatatt  
11281 gatattcaac aagtatttac tgaattgttt  
11341 tggggatctt cgagaaattt atttccccag  
11401 ccctggctat tgggacactt aagactctgg  
11461 gtttcagatt taaaggaattt gattagatta  
11521 ctagcaactt gggaaagctga ggtggggcaga  
11581 tgggcaacac ggtgaaaacc cgttctact  
11641 catgcacccatc taatcccgagc tttttgggg  
11701 aggagcaggat tggcggttggggcaagatgc  
11761 agatcccttc tccaaaaaaa aaaaaaaaaaa



16321 atctgcaccc tggagcaatg cagttagtct atgactcacg gatgctgtga atgctgtAAC  
 16381 tgcttcctt tctgcgtagt atgtgatcac ctacggTTT taaaaaccat tattgttAAA  
 16441 atcaaaacaa tcacagatAT gatttggta gtaactatca caaaggccAG acacaattCT  
 16501 tggtttatttt ttgagacggA gtttcaCTCA tcgcccAGGC tggagtgcGG tggtgcaATC  
 16561 tggctcaCTG caaactCTGc cttccgaggT caagtGATC tcctgcCTCC gtctccTGAG  
 16621 tagctgggat tacaggcact tgccaccaAG catggctaAT ttttGTTATT tttagtagAGA  
 16681 tggggTTCA ccatgttGc caggctggT tcgaactCCt gacCTCAGGT gatccacCCa  
 16741 cctcagcCTC ccaaaGtgc gggattacAG gcttGAGGCC cactgcACCT ggCCGGCCAC  
 16801 acacaattCC ttgcactTTA cctacCTTC ctaattCTCAT tctcACAGCA atctaaggAA  
 16861 ttataaaATT ttgtGCTTAT ttgttggcaAG agggAAactGA ggttCAGGGa gcatTTgAAA  
 16921 ttgtcaaaaAG cctcAGcaAG gaagagaaAG agccatGATG tgaatcaaAG tccactGGAG  
 16981 tggtaaAGAT tatCTatGGT gttgacCTT atctGCCCTT cacCTGACGT acttCTTTA  
 17041 agggAAatCAG tcatttaATAA agaaggAGtGAa gaagggAAA aaataAAAGGA agagAGtGAa tcaaggAAA  
 17101 ggaataaacAG agaaAGtGAa gaagggAAA aaataAAAGGA agagAGtGAa tcaaggAAA  
 17161 aaaactgatC gcattcCTAA tcattCTTTTAA aaacataATCC atcGcacaAA catacaacCT  
 17221 ttagcacttT aaaatGtACG gagTTTATTtT tacatATTAA catatGtaAT atgttaATAA  
 17281 ggggtgttAA catattAAAG cattaACTT AAAAATGGa atttGAAAGC caggcgcGGT  
 17341 ggctcACGCC tGtaatCCCA gcacttGGG aggCCAGAT ggggtggatCA cttgaggTCA  
 17401 ggaggtaaAG accaggcCTGG ccaacatGTTT AAAACCCCA ctctactAA aatacAAAAA  
 17461 ttagccaggT gtgggtGTGc acgcCTGTG tcccAGCTAC tcggggggCT gaagcaggAG  
 17521 aatcgcttGA acccGGGAGG tggatGTTGc agtGAGCCG gttcacacCA ctgcaCTCA  
 17581 gcctGGGCAA tagAGtGAGA ctgtCTCAA AAAAaaaaAAA AAAAaaaaAAA aaaaggAGTT  
 17641 ggagaaaaAT agccacacAG ctgttttAA ttaccaaAGA tccattGTTc atctGtGATG  
 17701 tgcagacCC tGtGCTAAcA gggatCTCA gagctGGGcC caatGGGACc tcaacAGACa  
 17761 cagccAGAAAT cacatCATGG aggttatGtG cagccCTGTT tcaaaaaAGCC aaatCCAGGG  
 17821 ctgggtGcAG tggctcatGC ctataatCC AGCATTtGG gaggCCAAAGG caggcggATC  
 17881 tcctgatCCC aggAGtGtA gatcAGCCTT ggcaACatGG agaaACCTT cctctacAAA  
 17941 aaaatacAAA acttagCTGG gtgtGgtGc atgtGCTGT ggtcccatCT actcaggAGG  
 18001 ctgaggGTGG aggatGGCTT caacAGAGCA agattCTGtC tcaaaaaAAA aaaaatCT  
 18061 ccactgcACT ccagcCTGGG ctataatCC AGCATTtGG gaggCCAAAGG caggcggATC  
 18121 gtaaaAGtAA aaaggGcTCC aaccatAGAT ttactGtATG aCCCCGtaAT tccactCCCA  
 18181 aaaataattC ctgatGtaATT tacacatAGAT ttactGtATG aCCCCGtaAT tccactCCCA  
 18241 ggttagGtGT tagtagGtGA gtttggCTG atataAAAtAC atatCCtAA gaactGAAA  
 18301 taggtGtCC aacaaaaAAAct tacacAGAAA ttgttcaGAGC agtTTATTtT gcaatAGtCA  
 18361 aaggGtAAA aacacacAAA tgcccatCAA cagatGAAAG gagaAAAATA tttggtatAT  
 18421 ccatacaatG caatGatATT cagccacACA aagAGAGAT gacatGtAC aactGtGGCT  
 18481 gaacCTCaaa aacacGACGc taagtGGAGG aggCCAAtCA caaaAGACCA tatattGtAC  
 18541 aatAGCATT ataggatGtG gacatAGTTT ttGAGAGCC atcattCCCA ctcactatGC  
 18601 ctgtGatAGT ttGTTTATGA agcaatAGAA aactAGtACa gtGACCACCC attaatttAT  
 18661 tcatttGTTt ttGTTGcGGG ttcaacAGAT acccaACTGAG cacttGtGT gtGcAGGcA  
 18721 ctatGCTGGG ctccAGAAAC actGAGtAA TAAGAGACT cgtGcCTGc tgaatACATC  
 18781 ttatGTTGG gagattCACT cttagGccCT tctcacaAAAG ggAGGcAAAGA tgccGAAAGA  
 18841 tcacGAGGAC tactGatGtG ttGAGCTGT gacaCTAAAG cAGCAGGGAC cAGTGGTCC  
 18901 ttggGTgACT tctGatGAAA gacaAGtACC ctcttCCAG gtacAAATGG aatataATGC  
 18961 tgcataTTAT ttcaAGAGAT tctaAGATAc tcAGGGTCCA ttcatcaAGG tccatAGTTA  
 19021 agaacACTG gcctAGAGAT ttGAGATG acttGAAAAA agaaATATAc taAGTAGAGA  
 19081 atgtactGCG agtGtCTGT cattGTTTCC aagtTTAAAC ttacttGGCT ggctGGAGT  
 19141 ggtggCTCAG ccGCTGAAcT ctGAGCATTtGGAGGccAA ggCggGtGGA tcacCTGAGG  
 19201 tcaggAGTTT gagaccAGTC tggccAAACAT ggtGAAACCC catCTTCTTtT AAAAATACAA  
 19261 aaattAGCCA ggcttGGTGG tggcGCTGT aatCCACCTtTtGTTGGAGG ctGAGGcAGG  
 19321 agaatCGTTT GAAcCTGGGA ggcAGGAGT gtGtGAGCC aaggTTGcAC cactGcACTC  
 19381 caccCTGGCA acAGAGCAAG actCCATCTC AAAAaaaaAAA AAAAaaaaACT tggcataACC  
 19441 accatGATGT ctaattGtAT gtGtCCACtT gactGGGcCA caggataCC AgatattATG  
 19501 ttaaaACATT tctCTAGTGT gtccatGAAG gCgtTTCTGG aggacattAA cgTTTcGATT  
 19561 agtactatGA gtaaaAGcAGA ctGccCTTCC caatGTTGGT ggCTTCATAC aatCTGTTGA  
 19621 aggCCTGAAT AAAAAtAAAAG gctGAGtGAG aaAGAActGC ctCTCTATGC ctGacttCAA  
 19681 gctGAGACAT cagCTTCTCC tGCCCTTGA ctcAGACTG gatATGGAAAt ggaACTGATG  
 19741 ccttttATC tcctGCTGT cAGCTGCTCA gactCAGACAt tGAactGTAC cattGTTGTT  
 19801 cctGGGTTtC gatGtCTGAG tCTCTGTAT ttcAtGAGtC aattCCTTAt taaaAtCT  
 19861 ttatataATC ctTTTATACAt atatacAGAA aatGtGtGT gttatGtGAc ttGtGtGTT  
 19921 atagatCTAT attGAtACT atataAtACt attatacACT gtattAGtAC acAGtGtATA  
 19981 gtacatacAC tataGtGTGT atatGtATAc tctatataCT atatGtTTA tataTATTt  
 20041 ttagtacATA taatGtATAG atatAGAAAG acacACCCAT aggtGtGTAT agatacAGAA  
 20101 acacacacCC atAGGtGTGT atAGGtACAG acAGACACAC ccatAGGTGT gtAtAGAtAC  
 20161 agacAGACAC ACCCATAGT gtGtaaAGAT acAGACAGAC acACCCATCG gtGtGtAtAG  
 20221 atacAGACAG acACACCCAT AGGTGtGTt AGTAtAGAt AGACACACCC AtAGGtGTGT  
 20281 gtatAGAt AGACACACCC GtagGtGTt AGTAtAGAt AGACACCCGT AGGTGtGTt  
 20341 agatAGtGAT atATCTCTCt CTATATATAt AATAGAtACAt tGtGtGtAtC tAtATCTAtA  
 20401 catAGAtACA cacACACATA tagtATTAGt tcaatttCtC cAGAGACTCC tactACAC  
 20461 atcAGAtCA ccaACAAcCA aAtCTTCTC AAtGTTTAt caccAGAAAG caAtTTtAGC  
 20521 ctGcACTGG AtAtTACAc cttGAAtAGA AAACATTAGA ggcCTGGAAAG catGGtGTt  
 20581 ttaatttCTT ttGtGCTTA cctatttCAA caatttCTGG actTTtTAAG acAGAAACTA  
 20641 ttcaACCAAC ttttGAAAAA tcaAAAGAAG gAAAtGAGtTt AAAGGTTTT acctGGAAtt  
 20701 caaacaAAAct aatGAAAGAt gacATTtATA AtAtTTAGAG aAtGAtTGGa AAAAtGGGcC  
 20761 ttcacttCAT gatAtTTAAAt aAtttGtGAt gtttttGGGT gtGtGtGtGAt tactGtAAtt



25321 atgcaatgg gatcccagaa gattgtataa ccatattta actgtacctt ttctatgttt  
 25381 gtatatgcaa atacttagca ttgttattaaa attgcctaca gtattcagga cagaaacatg  
 25441 ctgtacatgt ttgcgtccag ggagcaatat gctctaccta gcctaggagt atggcaggt  
 25501 ataccatcta ggattgtata ggtatactct atgatggtgg cacaatgaca aaatggccta  
 25561 acgaaggatt tctcagaaca caacccctc tctaagcaat gatgactgta tatgtaaatg  
 25621 tgtatgtcg tgaatatgtg tgtaatatact atgtacatgt atatttctat ttccataaagat  
 25681 agtacatgtt tttaaatgtc tgaattatact gcccccttcc attgtactga aagagatcta  
 25741 ttccatttct ttcccttcc tgagacagag tcttgcctcg tcacccacgc tggagtgcag  
 25801 tggcatgatc tgggtcact gcaatctcg cctcctgggt tcaaggatt ctccctgcctc  
 25861 agactccgtga gtacgtgcta ttacaggagt gtaccaccac aaccagctga tttttgtatt  
 25921 tttagcagaa atgagggtttt gtgatgttgg ccaggctggt ctgcgaactct tgacctcaag  
 25981 ctatccaccc gcctcagcca accaaagtgc tgcgattaca ggcgtgagcc actgtccag  
 26041 gctagatcta ttcaacttct acttcgataa aagtcaactac aagcctgtta ctccctgctt  
 26101 taaagcttc ggtccccctca ttgcctaca aacaacgtcc aggcctgttgc caaggccctt  
 26161 tccaaacgcag tccctgcctc aatcccttatac attttcttgc tgcattctgt tctggccctt  
 26221 tatgcacca aaaaattattt tctaggggacc agactgactc ttttcatccc tgaactccctt  
 26281 ttctatgtcg atccctctgc cagaaacatc ctccctggat actcatattt gtcttccctt  
 26341 gtctgaagga aacttccct aattatttgc ttgcctgc aagtaaagtt agataaaagtt  
 26401 agtcacccctc ttcttggta ctttgcgtgc gcaccgactt ttccgtaca aaactcattt  
 26461 tgattgattt gttcccaagt ctgtcttctg taaaagactt cgagttacat gagagtaagg  
 26521 actgggcctc aattttccctt ggatccccctg ggcttcacac agaatctgac agacccaagg  
 26581 tggaaagtccag gaaataagtg atgaatgaac aggctatctt ttctgatcca cacagcatga  
 26641 gggaaacaaa cacagaagggtt aggagagggtt gtgcgtctc ctccacccca accaataaac  
 26701 aaaaacaacc ttaaacccctt aaaaatgttatac agtctataca ggctggaaac tgggagaaga  
 26761 cagggtgtct tccctttttt gtttagtcttga gaatctgtat ggcacatgc ctgagcagga  
 26821 agtattaaaca caaacatcatc caaagtgc tttttttttt tattgtctgc cacatttaca  
 26881 aatttggaaatc gatcccttattt cagccagttt cgccaaagtt tctgcaaaatc gaacatagca  
 26941 aaagataggt ggtgccttcc agatcacctt tgattaaact cactgcattt gtcttcaaac  
 27001 aaaaatgttt ttttttccctt cccgcaacat acaatttttgc tcaagctaaaca ctaaatttaca  
 27061 caccactca gatttattt gataagaaca ttcaatgttgc taatttcaag aagatgactc  
 27121 ctataaattt ttataattttt ttattttagta ctttgggtttt taatcattaa gtacgtgaag  
 27181 actttgttagg tcaaaacccctt attaatgttgc acagataatctt atttttcaaa ctccctcct  
 27241 acccccttcc caaaatgttataa attaaatggaa agagatttttac acacacttgtt aagaacaaaag  
 27301 gtaataattt ggggggtggc cccactttcc taatcttgc agttagactg tcaatcatag  
 27361 tcctgactt tttatgttgc atcataacttgc tttttttttt taaaataatg ggtgttaat  
 27421 ttgagaagcc attacttccatc taagaaaataa aaacacataa cccatttttaa agttttgttta  
 27481 aaaaaaaaaaaa aaaaagctgc tttttttttt taaaataatc aaaaatataa aaaaatgttca  
 27541 gcctctaattt ccagccactt gggaggccga ggagggcaga tcaacctgagg tcgggggttcc  
 27601 aacaccgcg tggccaaaggg tggtaaaacc ccatttttttca taaaataatc aaaaatgttca  
 27661 gggagtgttgc gcatgtgc tttttttttt taaaataatc aaaaatataa aaaaatgttca  
 27721 ttgaaccccg ggggggggg tttttttttt taaaataatc aaaaatataa aaaaatgttca  
 27781 gtgacaacaaac aacatgttgc ttcaaaagaaa aaaaagaaaac aaatttagcaaa atacaggat  
 27841 cgttaggtgt tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc  
 27901 atgcataatgg aaaaatgggg tttttttttt taaaataatc aaaaatgttca tttttttttt  
 27961 ctaaaggaca gtcttcatct cttttttttt taaaataatc aaaaatgttca tttttttttt  
 28021 ttcaaaatccat tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc  
 28081 ttgataaactt gcatgttgc tttttttttt taaaataatc aaaaatgttca tttttttttt  
 28141 atctaaaatc aaaaatgttca tttttttttt taaaataatc aaaaatgttca tttttttttt  
 28201 agatgtttttt taaaataatc aaaaatgttca tttttttttt taaaataatc aaaaatgttca  
 28261 aaatgtttttt taaaataatc aaaaatgttca tttttttttt taaaataatc aaaaatgttca  
 28321 aaaaatataatc aaaaatgttca tttttttttt taaaataatc aaaaatgttca tttttttttt  
 28381 acagacaattt tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc  
 28441 ctttcggaaat aaaaaggcat tttttttttt taaaataatc aaaaatgttca tttttttttt  
 28501 caatgttgc tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc  
 28561 ttgttgcggg tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc  
 28621 cttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc aaaaatgttca  
 28681 cccactacac ccccccacccacg cttttttttt taaaataatc aaaaatgttca tttttttttt  
 28741 gcccccttc tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc  
 28801 atgagaagaa aataaaaaaaa ttcttgcctca tttttttttt taaaataatc aaaaatgttca  
 28861 cttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc aaaaatgttca  
 28921 ttgttgcgtt tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc  
 28981 gttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc aaaaatgttca  
 29041 atgcgttgc tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc  
 29101 ttgttgcgtt tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc  
 29161 cttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc aaaaatgttca  
 29221 cttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc aaaaatgttca  
 29281 tatgttgcgtt tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc  
 29341 cttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc aaaaatgttca  
 29401 cttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc aaaaatgttca  
 29461 ttgttgcgtt tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc  
 29521 tatgttgcgtt tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc  
 29581 tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc aaaaatgttca  
 29641 ttgttgcgtt tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc  
 29701 tttttttttt taaaataatc aaaaatgttca tttttttttt taaaataatc aaaaatgttca  
 29761 ccctcataatc aaaaatgttca tttttttttt taaaataatc aaaaatgttca tttttttttt

29821 gggtgtctcc taagtcaaga tggtcatttc atctgtggtc atccaaaagt taaaagggggg  
29881 ttggaggaac tgcttccaac aaggctcgct catgttgct ggcaatctgg tctgactgtt  
29941 ggaaggctga ttcccttgcata tgccgatctc cccatggtc tgtttagta tcctcgcaac  
30001 atgggtgtcta gcttctgcca caaggagtga tccaagagaa gcaaagcaga agtccaaatg  
30061 tattttgtca cctagcttca gaggtcatgc gctatcactt cccctgttag gttacacagg  
30121 acaatccctgt tcagagtata agagaaatatt actgcaatgt aaaaccagg aagtgagggt  
30181 cactagaggc catcttgacc tctggctacc acagaagtc atctccctt ctctagacag  
30241 tgccatgctc ttaagttt aaggttgaac tctaacattt caacaacaaa atgacactcaa  
30301 aatgaatgaaat atgagagaat gaaaagtcat cttgaagaaa tacaacatg tccaggcttca  
30361 agtcaacttca gattgtgggg ccagtattat acaaggatcc caaaagtgtc gtgttcaaa  
30421 cagctgagga atgaatgaag attacaggta cagaaccaa gaggcttag caatgatgt  
30481 gaagatgggc atgaagggtt taaatgaatatt tatttcataa atgttaaggat atataaaaac  
30541 tccagtctt taaaataat acattacttgc 30541 tccagtctt taaaataat acattacttgc  
30601 ataaaactt tgaacagttc atctacagcc ctaaaagttt ataaactggg cacaaaattt  
30661 agggggaggtc cacttcattt gaaaataaaaaaattt aattgttta tatttgaggt caccctgtat  
30721 ttggaaacgt attgactcgat ccaagattta ttgagcatct ctatgtcg gacactgtcc  
30781 taggttggac aaagtgtga atgagacaga taaagtccct caccgtctag tgggaaagat  
30841 gaaaaataaaa caaacacaaa cgtgcaatac agcttcaatc taaaagttt ataaactggg  
30901 tcttctgagg caaccaagca tttgagtcata ataggcaatg accttgcattt taatcattt  
30961 ccaattaaat aacttacaat gtcaacttggc tcagtaccg tacttctgcc tataagtaaa  
31021 ctctttttct gttagtccac ctataagaaa attctcatcc tctcttcata ctcagattaa  
31081 caagagcatt tcaaactatg tgataattt agtccctgtt gaaaattt gttatgacc  
31141 ctgggttacatc acatcgat tctgtgaattt ggttattgtt gactgtgtac ctaacagaaa  
31201 gacctacttca tagtgggtc aaaaagtcta atttctgaat gaccaagttt gagggtgaaa  
31261 gccatattgt taatgaactc atcaaggtaa ataccacaggc caagttctg gaaattaatg  
31321 cacctggcc tactgtccac acctaccctc tttcaataaa ctcacatgta atacattaaa  
31381 aaatttatct cttaggagctg caaaaagaaac actacatgca gagagaccca gaataactgag  
31441 agaggttgc gcacactccc gactgccatc cccgattcattt attcatgact gatcaataag  
31501 tgatcacaga aatcttccat gttgagccaa tattggaccca agtttgcattt tacttctgcc  
31561 aatagacact aagaactcat tagtgtgaaaat ttttttgcattt tacttctgcc  
31621 aatatctgtt tgcccaata agattgggtt gtttgcattt tacttctgcc  
31681 cccctgttcc acaggactg actggcagac agacacaatg agtttgcattt tacttctgcc  
31741 cattacataa agcttcaaca atggtaaca agacacaatg agtttgcattt tacttctgcc  
31801 tggaaactccca aggaatcgta aacaaatgggg gtttgcattt tacttctgcc  
31861 cgtttcattt caacattatt cttagcagta gtttgcattt tacttctgcc  
31921 tattgaagag tttatgagct ataaggactt gtttgcattt tacttctgcc  
31981 tatttgacat taataatcac tatttttgcattt tacttctgcc  
32041 gtggaaacact cgggatgttag tatttttgcattt tacttctgcc  
32101 tcatttcataa gggaaaatctt gatgggtt gtttgcattt tacttctgcc  
32161 gtgtgacac atggcttagt tatttttgcattt tacttctgcc  
32221 tatttttacac acacacactc acacacacactc gtttgcattt tacttctgcc  
32281 tgttagactaa aagccaaattt ctcactctat ctttttttgcattt tacttctgcc  
32341 tcgcttctgt tgcccaggct ggagtgcattt tacttctgcc  
32401 ctcccagggtt caagctattc tcctggccica gtttgcattt tacttctgcc  
32461 tgccatcaca cccggctaat ttttgcattt tacttctgcc  
32521 aggctgttgc cgaactccca accttaggtt gtttgcattt tacttctgcc  
32581 gatttacagg catgaggccac caccggccatc gtttgcattt tacttctgcc  
32641 tttggcttagt gtaagaacaa tataaaacccctt gtttgcattt tacttctgcc  
32701 tctagtcttagt ggagggaaac aaccatactgt gtttgcattt tacttctgcc  
32761 aaagtttgc aaggaaggcc tatctcaaaa gtttgcattt tacttctgcc  
32821 ttttttttgcattt agagggattt aggctctgtc gtttgcattt tacttctgcc  
32881 cgctcaactgc aagctccggcc tccgggttgc gtttgcattt tacttctgcc  
32941 agctgggact acaggccccc gccaccaggc gtttgcattt tacttctgcc  
33001 agatgggggtt tcaccgttgc agccaggata gtttgcattt tacttctgcc  
33061 cgcctggcc tcccaaagtg ctggattac gtttgcattt tacttctgcc  
33121 tgtgttttttgcattt gacaacttcg tgacttcgcata gtttgcattt tacttctgcc  
33181 acgccccaaatg gtgagtgctc ccagagactt gtttgcattt tacttctgcc  
33241 gtggccaaac taggagggtt acacagttaa gtttgcattt tacttctgcc  
33301 gggacaccaa gtagaccctg gggagctgtg gtttgcattt tacttctgcc  
33361 gcagttctgtt gcccaccat cctactgtctt gtttgcattt tacttctgcc  
33421 gcagcaatgtt attttcagca ctttacccc gtttgcattt tacttctgcc  
33481 aaaaaggctgtt actaactatctt ctgcaaggat gtttgcattt tacttctgcc  
33541 attaaggaaa tacagaatctt cagagggtt gtttgcattt tacttctgcc  
33601 aaaaggctgtt tgtaacttgc cctgttgcattt tacttctgcc  
33661 gtgggttcaac ctcatacttgc cctgttgcattt tacttctgcc  
33721 tttctgttcc agggtcagcc cttaggcaat gtttgcattt tacttctgcc  
33781 atactcctgc ctttccact ctgcaccctg gtttgcattt tacttctgcc  
33841 tacccttagg caacaccaca taaaggactt gtttgcattt tacttctgcc  
33901 cacctcatgc ccagtgcgtc ctgagctctc gtttgcattt tacttctgcc  
33961 gggccatcata ttttacacttgc gtttgcattt tacttctgcc  
34021 tccaaacgtt agcaataaca gtgcatttag gtttgcattt tacttctgcc  
34081 aacatgactt ggatgtttt tcaacattt gtttgcattt tacttctgcc  
34141 gattgggagc ccacgggttgc caaaatgtt gtttgcattt tacttctgcc  
34201 tcattggcat ttgctggata taaacccctt gtttgcattt tacttctgcc  
34261 ttttcttataattt gtttgcattt tacttctgcc

34321 ggggtcagggt ggtagcttgg ctatgtatgtca gatgtacttca gagggacccctc actcatatgc  
34381 caggcaccgaa caggctatca gctggggcaaa ccactctccc ttatgtggc caccatgctc  
34441 cagcagggtca gtcagggtt gttcgtagc tggtcacagg gtttaagca acgaaacaga  
34501 aagtcccagt atctgaccgc ttgtcatgcc tctgctgta, agatccact gggcaatgcc  
34561 agtcatacag gacagggtt gatgcgggtga gggaaatact gttttccctt ttcttctt  
34621 tttttttttt aaacaagcta ccattttac aatttacat acctcccca aatccctagcc  
34681 tttagtgtt attatttcca tcttataaaaaa gaagaaatctg agagttaaa aacatcccaa  
34741 agaaagctca gaataatgaa ctcagttgtg gctatgttca gttatccat aatccctt  
34801 caacacttag ggaagctgag gcccggaaat gctggatccaa gttatgttca gttatccat  
34861 gggcaacata gcaagaccct gcttccaca aaaaagaaaaa aacaaagaaa caagtttga  
34921 aaaaccagggt ttttgttgg gatttggaaat gctatgttca gttatgttca gttatccat  
34981 atataattta taatagatac tgatatgttta aatatacaca aacattaaa gtgtacat  
35041 cagaaagaaa tataaataata cttaaaattat aatatacaca aacattaaa gtgtacat  
35101 gatagggtgt tttgtatataat aatatacaca aacattaaa gtgtacat  
35161 cttagaagcaa tgacactcca gcaactatgaa acacatattt ctaaactctg ttgagaagg  
35221 atatgattct ataataaaaac gaccaaggcc ggcgaacttag cacctatgat ttagttctaa  
35281 aggccggaa gtcggatcc acagtgtaaa tccatggaga atgatgttca tccatggaga  
35341 gctcaaaaaaa gaacggggca tttgtatataat aatatacaca aacattaaa gtgtacat  
35401 gggccaaaggc cggaaacatt tgacgacccaa tccatggaga atgatgttca tccatggaga  
35461 tacagaataaa actaaatatt cacgtatcca aatatacaca aacattaaa gtgtacat  
35521 cttccatgtaa gaagaattcc caccaaaaaaa gaccaaggatcc gtcggatcc  
35581 catgagaataa ccacagtatt aatcttgc aatatacaca aacattaaa gtgtacat  
35641 atggatgaaa attagagaag tagtcccaaa aatatacaca aacattaaa gtgtacat  
35701 aaggaaaaaaa atgatctttt acagtggaaa tccatggaga atgatgttca tccatggaga  
35761 tcaaggctaa catcactatg aataagatata aatatacaca aacattaaa gtgtacat  
35821 cccagaatgtg gcacatcaat tttgtatataat aatatacaca aacattaaa gtgtacat  
35881 caagagaaca catcagacaa accccaaatata aatatacaca aacattaaa gtgtacat  
35941 tcttcaaaaaa tttcaaggcc attaaaaaaa aatatacaca aacattaaa gtgtacat  
36001 ggggacttggaa gagatatgtat aataaaatgtcc aatatacaca aacattaaa gtgtacat  
36061 ggacacaccg tcaatccaa ataaaatgtcc aatatacaca aacattaaa gtgtacat  
36121 agcatcataaa caagggttac ttctcgttgc aatatacaca aacattaaa gtgtacat  
36181 agctgagtgtaa agactacatg gggaaatctt aatatacaca aacattaaa gtgtacat  
36241 aacattttt tcaaaaaattt aaaaactggaa aatatacaca aacattaaa gtgtacat  
36301 ccacagctcc ttgtacttgc gtcgttgc aatatacaca aacattaaa gtgtacat  
36361 tgctttccat cctgtcgttgc gctcccccgc aatatacaca aacattaaa gtgtacat  
36421 ctggatttagg ccacccttc caaatgtt aatatacaca aacattaaa gtgtacat  
36481 ggcataaaacc catctacattt aatggggaa aatatacaca aacattaaa gtgtacat  
36541 gcagaatattt cactcttca ttcaactctca aatatacaca aacattaaa gtgtacat  
36601 gcttataatgtc ttatcttttgc attccattaa aatatacaca aacattaaa gtgtacat  
36661 ttcaaccatt ttgtcaattt aatgtgtt aatatacaca aacattaaa gtgtacat  
36721 taccttgttca attacttact taatcatgc aatatacaca aacattaaa gtgtacat  
36781 actataaaattt atggacttattt acgcagcaaa aatatacaca aacattaaa gtgtacat  
36841 aaacccttgc ttttggggaa aattttactt aatatacaca aacattaaa gtgtacat  
36901 tgcttaaggctt gtaaaagaga atccatgagc aatatacaca aacattaaa gtgtacat  
36961 agttgacttcc agtttccagg ccgcttcaaa aatatacaca aacattaaa gtgtacat  
37021 gatatttcatg tcagaatggc cgtgttgc aatatacaca aacattaaa gtgtacat  
37081 aggctggaaa ctttggggat cttcacctt aatatacaca aacattaaa gtgtacat  
37141 taatgttcat ttgtggccat aatagcagac aatatacaca aacattaaa gtgtacat  
37201 ttggattctttt ttttttttta agaccgttcc aatatacaca aacattaaa gtgtacat  
37261 aaaaaaaaaaaac acagcaagat aaaaaaaaaat aatatacaca aacattaaa gtgtacat  
37321 gacaacgacc ctttggaaatg aaggttttcc aatatacaca aacattaaa gtgtacat  
37381 tgcgtgtgtt ccgcattgtt gggagacagcc aatatacaca aacattaaa gtgtacat  
37441 ctgcctcaat aggccagtct ttgttgc aatatacaca aacattaaa gtgtacat  
37501 ccactagtc tggccactt ggtgtatgtc aatatacaca aacattaaa gtgtacat  
37561 gggtcagctt acacgggtac actgttagac aatatacaca aacattaaa gtgtacat  
37621 aaaaataataac aagactaaaa aaggaggatg aatatacaca aacattaaa gtgtacat  
37681 gcagacagttt gcccaggat aatgtctgc aatatacaca aacattaaa gtgtacat  
37741 ttgaggatataat ttttttttta agaccgttcc aatatacaca aacattaaa gtgtacat  
37801 ctgcggtctt tccctccaca ttgtgttgc aatatacaca aacattaaa gtgtacat  
37861 ccattgttta tttccctgtt ttgtgttgc aatatacaca aacattaaa gtgtacat  
37921 taatttatcac ttgttaataga gaaaggcttcc aatatacaca aacattaaa gtgtacat  
37981 ttcatcgtaa cccactgtt ccacacggca aatatacaca aacattaaa gtgtacat  
38041 catatgttagtataaagggatgtt aatatacaca aacattaaa gtgtacat  
38101 aaaaataataac tcaacttgc ttttttttta agaccgttcc aatatacaca aacattaaa gtgtacat  
38161 ctctcttgggaa ctaagaatag cacaactaaa aatatacaca aacattaaa gtgtacat  
38221 aggtggggaa tcacttacag ttttttttta agaccgttcc aatatacaca aacattaaa gtgtacat  
38281 acagtatcggt tctacaaggc acccagcgctc aatatacaca aacattaaa gtgtacat  
38341 agcttattta taaaatgtt accaaattcc aatatacaca aacattaaa gtgtacat  
38401 gcatgttggaa tacatcatct gggttttggc aatatacaca aacattaaa gtgtacat  
38461 gcttttcgggt gttgtctgc cattagggac aatatacaca aacattaaa gtgtacat  
38521 gcagcgttcc agagaaaaaca aataaaaat aatatacaca aacattaaa gtgtacat  
38581 ctgaagggtt gtaaaatccaa ctatgtt aatatacaca aacattaaa gtgtacat  
38641 aaaaatatttttgc atgcacaggat ttttttttta agaccgttcc aatatacaca aacattaaa gtgtacat  
38701 cattatttgc atccatgttgc agccacgggag aatatacaca aacattaaa gtgtacat  
38761 ttttttttttgc ttttttttgc atttttttta agaccgttcc aatatacaca aacattaaa gtgtacat



43321 taatacagca atctcatgca cccttgatcc agtttccccc ataggtata ttcacaaaa  
43381 ctcttagtacg atttcacaac cagggaaattg acatttgagac agtcaagatg cagaacattt  
43441 ccatctatac caggatctc cagttgtgtt ttatatccca ctctggttcc cactcacc  
43501 ccactcctct ccagtcctg gcaatcaactt atctgttgtc ttccctttt ttctctttt  
43561 ttctctttt agatggaga ccactctgtt gcccaggctg gagatcaggg gacatctg  
43621 ggctcaactgc aacctctgac tcccggttc aagcaattct cctgtctcag cttcccgag  
43681 tgctgggtat tacagggtg tgcttaccac gcccggcta actttttgtt ttttttagt  
43741 gagatgggtt ttcacaatat tggtcaggct ggtctggaa tccagacctc aggtgaccca  
43801 tctgccttgg cctcccaaag tgctgggatt acaggagtga gccaccgtgc ccagccatct  
43861 gtttcttattt tttataattt tctaatttca agaacattat gtaaatggaa tcctaccaat  
43921 gtaatctttt gggattggct ttttactctt tttatagtc atttgcagcc aaagaaaata  
43981 ttcttattgt gtatacgtgt aagtctcaac attttaagat gcaactgtgt cttagttct  
44041 ctaagaatca acatgcagga ggcctaaag cctcctctga agtcaagctc tctggtcct  
44101 gtgtcaactgt cactctgca cggcatggcc caactacct gtctttttt gacgctcg  
44161 tgcttctgtt ggaatgacca aggcacagcc ctccacgtac cctgcttgc atccctctgc  
44221 ttccaccacca cccaccacaa cctgaaaaggc taccttccaa tggtcatgc gctgacaga  
44281 gttgaaggag gaggctctca agtaccagc cttccccaca accccagaga caaagcagaa  
44341 ggcacacagc caagatgggt tcctggctc taactgtgc agtcttctc aaaatgagaa  
44401 tagccttggg aggtgacaaag acctttggag aaaccttgg aacaacacaa agtaaattt  
44461 ctcattcaag ctttctctgc ctttgagcag gtaggtccct gggactccat ttctcatct  
44521 gtaaaatggg gacaataata cttaccttta tatttattga tatttctaca caggatgact  
44581 atgaaaatttta agtaaaattaa taaatataaag actcttaca ccatgccaa atcttaggaag  
44641 tataatagata tggtggat ttttatttaca aattaccacaa ttttccatc  
44701 tcaacttggat ttcttaatcac tcaatctttt ttttccatc  
44761 aatatttggt tccaatataa agatttctt ttttccatc  
44821 ttttattttttt cttcaatctt gcaactgttag ttttccatc  
44881 cccactcctg agaagtgttca aggttttagt ttttccatc  
44941 aagtgcacatc catatttgc catttttgc ttttccatc  
45001 gaattgcctt gatgcataat cacacagaat ttttccatc  
45061 ctgggtgagg gtttaggaat aaaggagtca ttttccatc  
45121 gaaatggaaa tgaaaatcgc gttggaaatg ttttccatc  
45181 acatttttac atagctcaag ttttccatc  
45241 catgcagcta aacattttt ttttccatc  
45301 cttcttcttc tccccctcc ctaagcagca ttttccatc  
45361 ttaatataat ctgggtctg gtttccattt ttttccatc  
45421 agagaacaaat cttcaactctc cctgtttctc ttttccatc  
45481 atattttgtc gccaacccctt gactctgaaa ttttccatc  
45541 tactgtctggg ccccttatgc taagagattt ttttccatc  
45601 caaacacaaag gggtttttgg ttttccatc  
45661 tcctgggttc cagcctcatc ttttccatc  
45721 cagtttttgc gtttccatc ttttccatc  
45781 gatggtagca agaattaaat gaggtactgt ttttccatc  
45841 gaggaatgtt cattctttt ttttccatc  
45901 ggcagaagtgt ttttccatc  
45961 aggtgggtcc acagtagtat ttttccatc  
46021 gaaaccactt cccaaatcac agagggggcc ttttccatc  
46081 aaatggacca taagggtttt cttcttgc  
46141 cagcagttaca acttgcacaa gagccgtccc ttttccatc  
46201 tgcaaaaggaa gagtaatct aactgtttaaa ttttccatc  
46261 atacccttac aaagaagagg ccaggcagta ttttccatc  
46321 tacaaggctca ctttctcaac gaaatcaata  
46381 ctatgttattt tggagttgaa atacatttgc  
46441 tggcagctta ggttagtaaca taatttttgc  
46501 aggatcaacatc ttttccatc  
46561 atttcagatt ttttccatc  
46621 ttttccatc  
46681 gtttccatc  
46741 gtttccatc  
46801 ataataactg atcttagccaa ttttccatc  
46861 aactgtgtga acatttttgc  
46921 cacccttctct ttccatc  
46981 ttggatggat attttccctt taaatgttgc  
47041 ctttccatc  
47101 ttggatggat tagttaaagg gataaaatgttgc  
47161 ttggatggat ttttccatc  
47221 ataaagacacaa ctttccatc  
47281 attcaagccca atatatttgc  
47341 ctttccatc  
47401 gaaacaaaaaa gaagactgga aatacagttt  
47461 gacagccctt caataattac  
47521 gatttacca aacagtttgc  
47581 gtttccatc  
47641 acatagcaca ctttccatc  
47701 ctatcaaattt ttttccatc  
47761 caagaaagat caagaatttcc  
47821 atttttccatc  
47881 ttttccatc  
47941 ttttccatc  
48001 ttttccatc  
48061 ttttccatc  
48121 ttttccatc  
48181 ttttccatc  
48241 ttttccatc  
48301 ttttccatc  
48361 ttttccatc  
48421 ttttccatc  
48481 ttttccatc  
48541 ttttccatc  
48601 ttttccatc  
48661 ttttccatc  
48721 ttttccatc  
48781 ttttccatc  
48841 ttttccatc  
48901 ttttccatc  
48961 ttttccatc  
49021 ttttccatc  
49081 ttttccatc  
49141 ttttccatc  
49201 ttttccatc  
49261 ttttccatc  
49321 ttttccatc  
49381 ttttccatc  
49441 ttttccatc  
49501 ttttccatc  
49561 ttttccatc  
49621 ttttccatc  
49681 ttttccatc  
49741 ttttccatc  
49801 ttttccatc  
49861 ttttccatc  
49921 ttttccatc  
49981 ttttccatc  
50001 ttttccatc  
50061 ttttccatc  
50121 ttttccatc  
50181 ttttccatc  
50241 ttttccatc  
50301 ttttccatc  
50361 ttttccatc  
50421 ttttccatc  
50481 ttttccatc  
50541 ttttccatc  
50601 ttttccatc  
50661 ttttccatc  
50721 ttttccatc  
50781 ttttccatc  
50841 ttttccatc  
50901 ttttccatc  
50961 ttttccatc  
51021 ttttccatc  
51081 ttttccatc  
51141 ttttccatc  
51201 ttttccatc  
51261 ttttccatc  
51321 ttttccatc  
51381 ttttccatc  
51441 ttttccatc  
51501 ttttccatc  
51561 ttttccatc  
51621 ttttccatc  
51681 ttttccatc  
51741 ttttccatc  
51801 ttttccatc  
51861 ttttccatc  
51921 ttttccatc  
51981 ttttccatc  
52001 ttttccatc  
52061 ttttccatc  
52121 ttttccatc  
52181 ttttccatc  
52241 ttttccatc  
52301 ttttccatc  
52361 ttttccatc  
52421 ttttccatc  
52481 ttttccatc  
52541 ttttccatc  
52601 ttttccatc  
52661 ttttccatc  
52721 ttttccatc  
52781 ttttccatc  
52841 ttttccatc  
52901 ttttccatc  
52961 ttttccatc  
53021 ttttccatc  
53081 ttttccatc  
53141 ttttccatc  
53201 ttttccatc  
53261 ttttccatc  
53321 ttttccatc  
53381 ttttccatc  
53441 ttttccatc  
53501 ttttccatc  
53561 ttttccatc  
53621 ttttccatc  
53681 ttttccatc  
53741 ttttccatc  
53801 ttttccatc  
53861 ttttccatc  
53921 ttttccatc  
53981 ttttccatc  
54041 ttttccatc  
54101 ttttccatc  
54161 ttttccatc  
54221 ttttccatc  
54281 ttttccatc  
54341 ttttccatc  
54401 ttttccatc  
54461 ttttccatc  
54521 ttttccatc  
54581 ttttccatc  
54641 ttttccatc  
54701 ttttccatc  
54761 ttttccatc  
54821 ttttccatc  
54881 ttttccatc  
54941 ttttccatc  
55001 ttttccatc  
55061 ttttccatc  
55121 ttttccatc  
55181 ttttccatc  
55241 ttttccatc  
55301 ttttccatc  
55361 ttttccatc  
55421 ttttccatc  
55481 ttttccatc  
55541 ttttccatc  
55601 ttttccatc  
55661 ttttccatc  
55721 ttttccatc  
55781 ttttccatc  
55841 ttttccatc  
55901 ttttccatc  
55961 ttttccatc  
56021 ttttccatc  
56081 ttttccatc  
56141 ttttccatc  
56201 ttttccatc  
56261 ttttccatc  
56321 ttttccatc  
56381 ttttccatc  
56441 ttttccatc  
56501 ttttccatc  
56561 ttttccatc  
56621 ttttccatc  
56681 ttttccatc  
56741 ttttccatc  
56801 ttttccatc  
56861 ttttccatc  
56921 ttttccatc  
56981 ttttccatc  
57001 ttttccatc  
57061 ttttccatc  
57121 ttttccatc  
57181 ttttccatc  
57241 ttttccatc  
57301 ttttccatc  
57361 ttttccatc  
57421 ttttccatc  
57481 ttttccatc  
57541 ttttccatc  
57601 ttttccatc  
57661 ttttccatc  
57721 ttttccatc  
57781 ttttccatc  
57841 ttttccatc  
57901 ttttccatc  
57961 ttttccatc  
58021 ttttccatc  
58081 ttttccatc  
58141 ttttccatc  
58201 ttttccatc  
58261 ttttccatc  
58321 ttttccatc  
58381 ttttccatc  
58441 ttttccatc  
58501 ttttccatc  
58561 ttttccatc  
58621 ttttccatc  
58681 ttttccatc  
58741 ttttccatc  
58801 ttttccatc  
58861 ttttccatc  
58921 ttttccatc  
58981 ttttccatc  
59001 ttttccatc  
59061 ttttccatc  
59121 ttttccatc  
59181 ttttccatc  
59241 ttttccatc  
59301 ttttccatc  
59361 ttttccatc  
59421 ttttccatc  
59481 ttttccatc  
59541 ttttccatc  
59601 ttttccatc  
59661 ttttccatc  
59721 ttttccatc  
59781 ttttccatc  
59841 ttttccatc  
59901 ttttccatc  
59961 ttttccatc  
60021 ttttccatc  
60081 ttttccatc  
60141 ttttccatc  
60201 ttttccatc  
60261 ttttccatc  
60321 ttttccatc  
60381 ttttccatc  
60441 ttttccatc  
60501 ttttccatc  
60561 ttttccatc  
60621 ttttccatc  
60681 ttttccatc  
60741 ttttccatc  
60801 ttttccatc  
60861 ttttccatc  
60921 ttttccatc  
60981 ttttccatc  
61001 ttttccatc  
61061 ttttccatc  
61121 ttttccatc  
61181 ttttccatc  
61241 ttttccatc  
61301 ttttccatc  
61361 ttttccatc  
61421 ttttccatc  
61481 ttttccatc  
61541 ttttccatc  
61601 ttttccatc  
61661 ttttccatc  
61721 ttttccatc  
61781 ttttccatc  
61841 ttttccatc  
61901 ttttccatc  
61961 ttttccatc  
62021 ttttccatc  
62081 ttttccatc  
62141 ttttccatc  
62201 ttttccatc  
62261 ttttccatc  
62321 ttttccatc  
62381 ttttccatc  
62441 ttttccatc  
62501 ttttccatc  
62561 ttttccatc  
62621 ttttccatc  
62681 ttttccatc  
62741 ttttccatc  
62801 ttttccatc  
62861 ttttccatc  
62921 ttttccatc  
62981 ttttccatc  
63001 ttttccatc  
63061 ttttccatc  
63121 ttttccatc  
63181 ttttccatc  
63241 ttttccatc  
63301 ttttccatc  
63361 ttttccatc  
63421 ttttccatc  
63481 ttttccatc  
63541 ttttccatc  
63601 ttttccatc  
63661 ttttccatc  
63721 ttttccatc  
63781 ttttccatc  
63841 ttttccatc  
63901 ttttccatc  
63961 ttttccatc  
64021 ttttccatc  
64081 ttttccatc  
64141 ttttccatc  
64201 ttttccatc  
64261 ttttccatc  
64321 ttttccatc  
64381 ttttccatc  
64441 ttttccatc  
64501 ttttccatc  
64561 ttttccatc  
64621 ttttccatc  
64681 ttttccatc  
64741 ttttccatc  
64801 ttttccatc  
64861 ttttccatc  
64921 ttttccatc  
64981 ttttccatc  
65001 ttttccatc  
65061 ttttccatc  
65121 ttttccatc  
65181 ttttccatc  
65241 ttttccatc  
65301 ttttccatc  
65361 ttttccatc  
65421 ttttccatc  
65481 ttttccatc  
65541 ttttccatc  
65601 ttttccatc  
65661 ttttccatc  
65721 ttttccatc  
65781 ttttccatc  
65841 ttttccatc  
65901 ttttccatc  
65961 ttttccatc  
66021 ttttccatc  
66081 ttttccatc  
66141 ttttccatc  
66201 ttttccatc  
66261 ttttccatc  
66321 ttttccatc  
66381 ttttccatc  
66441 ttttccatc  
66501 ttttccatc  
66561 ttttccatc  
66621 ttttccatc  
66681 ttttccatc  
66741 ttttccatc  
66801 ttttccatc  
66861 ttttccatc  
66921 ttttccatc  
66981 ttttccatc  
67001 ttttccatc  
67061 ttttccatc  
67121 ttttccatc  
67181 ttttccatc  
67241 ttttccatc  
67301 ttttccatc  
67361 ttttccatc  
67421 ttttccatc  
67481 ttttccatc  
67541 ttttccatc  
67601 ttttccatc  
67661 ttttccatc  
67721 ttttccatc  
67781 ttttccatc  
67841 ttttccatc  
67901 ttttccatc  
67961 ttttccatc  
68021 ttttccatc  
68081 ttttccatc  
68141 ttttccatc  
68201 ttttccatc  
68261 ttttccatc  
68321 ttttccatc  
68381 ttttccatc  
68441 ttttccatc  
68501 ttttccatc  
68561 ttttccatc  
68621 ttttccatc  
68681 ttttccatc  
68741 ttttccatc  
68801 ttttccatc  
68861 ttttccatc  
68921 ttttccatc  
68981 ttttccatc  
69001 ttttccatc  
69061 ttttccatc  
69121 ttttccatc  
69181 ttttccatc  
69241 ttttccatc  
69301 ttttccatc  
69361 ttttccatc  
69421 ttttccatc  
69481 ttttccatc  
69541 ttttccatc  
69601 ttttccatc  
69661 ttttccatc  
69721 ttttccatc  
69781 ttttccatc  
69841 ttttccatc  
69901 ttttccatc  
69961 ttttccatc  
70021 ttttccatc  
70081 ttttccatc  
70141 ttttccatc  
70201 ttttccatc  
70261 ttttccatc  
70321 ttttccatc  
70381 ttttccatc  
70441 ttttccatc  
70501 ttttccatc  
70561 ttttccatc  
70621 ttttccatc  
70681 ttttccatc  
70741 ttttccatc  
70801 ttttccatc  
70861 ttttccatc  
70921 ttttccatc  
70981 ttttccatc  
71001 ttttccatc  
71061 ttttccatc  
71121 ttttccatc  
71181 ttttccatc  
71241 ttttccatc  
71301 ttttccatc  
71361 ttttccatc  
71421 ttttccatc  
71481 ttttccatc  
71541 ttttccatc  
71601 ttttccatc  
71661 ttttccatc  
71721 ttttccatc  
71781 ttttccatc  
71841 ttttccatc  
71901 ttttccatc  
71961 ttttccatc  
72021 ttttccatc  
72081 ttttccatc  
72141 ttttccatc  
72201 ttttccatc  
72261 ttttccatc  
72321 ttttccatc  
72381 ttttccatc  
72441 ttttccatc  
72501 ttttccatc  
72561 ttttccatc  
72621 ttttccatc  
72681 ttttccatc  
72741 ttttccatc  
72801 ttttccatc  
72861 ttttccatc  
72921 ttttccatc  
72981 ttttccatc  
73001 ttttccatc  
73061 ttttccatc  
73121 ttttccatc  
73181 ttttccatc  
73241 ttttccatc  
73301 ttttccatc  
73361 ttttccatc  
73421 ttttccatc  
73481 ttttccatc  
73541 ttttccatc  
73601 ttttccatc  
73661 ttttccatc  
73721 ttttccatc  
73781 ttttccatc  
73841 ttttccatc  
73901 ttttccatc  
73961 ttttccatc  
74021 ttttccatc  
74081 ttttccatc  
74141 ttttccatc  
74201 ttttccatc  
74261 ttttccatc  
74321 ttttccatc  
74381 ttttccatc  
74441 ttttccatc  
74501 ttttccatc  
74561 ttttccatc  
74621 ttttccatc  
74681 ttttccatc  
74741 ttttccatc  
74801 ttttccatc  
74861 ttttccatc  
74921 ttttccatc  
74981 ttttccatc  
75001 ttttccatc  
75061 ttttccatc  
75121 ttttccatc  
75181 ttttccatc  
75241 ttttccatc  
75301 ttttccatc  
75361 ttttccatc  
75421 ttttccatc  
75481 ttttccatc  
75541 ttttccatc  
75601 ttttccatc  
75661 ttttccatc  
75721 ttttccatc  
75781 ttttccatc  
75841 ttttccatc  
75901 ttttccatc  
75961 ttttccatc  
76021 ttttccatc  
76081 ttttccatc  
76141 ttttccatc  
76201 ttttccatc  
76261 ttttccatc  
76321 ttttccatc  
76381 ttttccatc  
76441 ttttccatc  
76501 ttttccatc  
76561 ttttccatc  
76621 ttttccatc  
76681 ttttccatc  
76741 ttttccatc  
76801 ttttccatc  
76861 ttttccatc  
76921 ttttccatc  
76981 ttttccatc  
77001 ttttccatc  
77061 ttttccatc  
77121 ttttccatc  
77181 ttttccatc  
77241 ttttccatc  
77301 ttttccatc  
77361 ttttccatc  
77421 ttttccatc  
77481 ttttccatc  
77541 ttttccatc  
77601 ttttccatc  
77661 ttttccatc  
77721 ttttccatc  
77781 ttttccatc  
77841 ttttccatc  
77901 ttttccatc  
77961 ttttccatc  
78021 ttttccatc  
78081 ttttccatc  
78141 ttttccatc  
78201 ttttccatc  
78261 ttttccatc  
78321 ttttccatc  
78381 ttttccatc  
78441 ttttccatc  
78501 ttttccatc  
78561 ttttccatc  
78621 ttttccatc  
78681 ttttccatc  
78741 ttttccatc  
78801 ttttccatc  
78861 ttttccatc  
78921 ttttccatc  
78981 ttttccatc  
79001 ttttccatc  
79061 ttttccatc  
79121 ttttccatc  
79181 ttttccatc  
79241 ttttccatc  
79301 ttttccatc  
79361 ttttccatc  
79421 ttttccatc  
79481 ttttccatc  
79541 ttttccatc  
79601 ttttccatc  
79661 ttttccatc  
79721 ttttccatc  
79781 ttttccatc  
79841 ttttccatc  
79901 ttttccatc  
79961 ttttccatc  
80021 ttttccatc  
80081 ttttccatc  
80141 ttttccatc  
80201 ttttccatc  
80261 ttttccatc  
80321 ttttccatc  
80381 ttttccatc  
80441 ttttccatc  
80501 ttttccatc  
80561 ttttccatc  
80621 ttttccatc  
80681 ttttccatc  
80741 ttttccatc  
80801 ttttccatc  
80861 ttttccatc  
80921 ttttccatc  
80981 ttttccatc  
81001 ttttccatc  
81061 ttttccatc  
81121 ttttccatc  
81181 ttttccatc  
81241 ttttccatc  
81301 ttttccatc  
81361 ttttccatc  
81421 ttttccatc  
81481 ttttccatc  
81541 ttttccatc  
81601 ttttccatc  
81661 ttttccatc  
81721 ttttccatc  
81781 ttttccatc  
81841 ttttccatc  
81901 ttttccatc  
81961 ttttccatc  
82021 ttttccatc  
82081 ttttccatc  
82141 ttttccatc  
82201 ttttccatc  
82261 ttttccatc  
82321 ttttccatc  
82381 ttttccatc  
82441 ttttccatc  
82501 ttttccatc  
82561 ttttccatc  
82621 ttttccatc  
82681 ttttccatc  
82741 ttttccatc  
82801 ttttccatc  
82861 ttttccatc  
82921 ttttccatc  
82981 ttttccatc  
83001 ttttccatc  
83061 ttttccatc  
83121 ttttccatc  
83181 ttttccatc  
83241 ttttccatc  
83301 ttttccatc  
83361 ttttccatc  
83421 ttttccatc  
83481 ttttccatc  
83541 ttttccatc  
83601 ttttccatc  
83661 ttttccatc  
83721 ttttccatc  
83781 ttttccatc  
83841 ttttccatc  
83901 ttttccatc  
83961 ttttccatc  
84021 ttttccatc  
84081 ttttccatc  
84141 ttttccatc  
84201 ttttccatc  
84261 ttttccatc  
84321 ttttccatc  
84381 ttttccatc  
84441 ttttccatc  
84501 ttttccatc  
84561 ttttccatc  
84621 ttttccatc  
84681 ttttccatc  
84741 ttttccatc  
84801 ttttccatc  
84861 ttttccatc  
84921 ttttccatc  
84981 ttttccatc  
85001 ttttccatc  
85061 ttttccatc  
85121 ttttccatc  
85181 ttttccatc  
85241 ttttccatc  
85301 ttttccatc  
85361 ttttccatc  
85421 ttttccatc  
85481 ttttccatc  
85541 ttttccatc  
85601 ttttccatc  
85661 ttttccatc  
85721 ttttccatc  
85781 ttttccatc  
85841 ttttccatc  
85901 ttttccatc  
85961 ttttccatc  
86021 ttttccatc  
86081 ttttccatc  
86141 ttttccatc  
86201 ttttccatc  
86261 ttttccatc  
86321 ttttccatc  
86381 ttttccatc  
86441 ttttccatc  
86501 ttttccatc  
86561 ttttccatc  
86621 ttttccatc  
86681 ttttccatc  
86741 ttttccatc  
86801 ttttccatc  
86861 ttttccatc  
86921 ttttccatc  
86981 ttttccatc  
87001 ttttccatc  
87061 ttttccatc  
87121 ttttccatc  
87181 ttttccatc  
87241 ttttccatc  
87301 ttttccatc  
87361 ttttccatc  
87421 ttttccatc  
87481 ttttccatc  
87541 ttttccatc  
87601 ttttccatc  
87661 ttttccatc  
87721 ttttccatc  
87781 ttttccatc  
87841 ttttccatc  
87901 ttttccatc  
87961 ttttccatc  
88021 ttttccatc  
88081 ttttccatc  
88141 ttttccatc  
88201 ttttccatc  
88261 ttttccatc  
88321 ttttccatc  
88381 ttttccatc  
88441 ttttccatc  
88501 ttttccatc  
88561 ttttccatc  
88621 ttttccatc  
88681 ttttccatc  
88741 ttttccatc  
88801 ttttccatc  
88861 ttttccatc  
88921 ttttccatc  
88981 ttttccatc  
89001 ttttccatc  
89061 ttttccatc  
89121 ttttccatc  
89181 ttttccatc  
89241 ttttccatc  
89301 ttttccatc  
89361 ttttccatc  
89421 ttttccatc  
89481 ttttccatc  
89541 ttttccatc  
89601 ttttccatc  
89661 ttttccatc  
89721 ttttccatc  
89781 ttttccatc  
89841 ttttccatc  
89901 ttttccatc  
89961 ttttccatc  
90021 ttttccatc  
90081 ttttccatc  
90141 ttttccatc  
90201 ttttccatc  
90261 ttttccatc  
90321 ttttccatc  
90381 ttttccatc  
90441 ttttccatc  
90501 ttttccatc  
90561 ttttccatc  
90621 ttttccatc  
90681 ttttccatc  
90741 ttttccatc  
90801 ttttccatc  
90861 ttttccatc  
90921 ttttccatc  
90981 ttttccatc  
91001 ttttccatc  
91061 ttttccatc  
91121 ttttccatc  
91181 ttttccatc  
91241 ttttccatc  
91301 ttttccatc  
91361 ttttccatc  
91421 ttttccatc  
91481 ttttccatc  
91541 ttttccatc  
91601 ttttccatc  
91661 ttttccatc  
91721 ttttccatc  
91781 ttttccatc  
91841 ttttccatc  
91901 ttttccatc  
91961 ttttccatc  
92021 ttttccatc  
92081 ttttccatc  
92141 ttttccatc  
92201 ttttccatc  
92261 ttttccatc  
92321 ttttccatc  
92381 ttttccatc  
92441 ttttccatc  
92501 ttttccatc  
92561 ttttccatc  
92621 ttttccatc  
92681 ttttccatc  
92741 ttttccatc  
92801 ttttccatc  
92861 ttttccatc  
92921 ttttccatc  
92981 ttttccatc  
93001 ttttccatc  
93061 ttttccatc  
93121 ttttccatc  
93181 ttttccatc  
93241 ttttccatc  
93301 ttttccatc  
93361 ttttccatc  
93421 ttttccatc  
93481 ttttccatc  
93541 ttttccatc  
93601 ttttccatc  
93661 ttttccatc  
93721 ttttccatc  
93781 ttttccatc  
93841 ttttccatc  
93901 ttttccatc  
93961 ttttccatc  
94021 ttttccatc  
94081 ttttccatc  
94141 ttttccatc  
94201 ttttccatc  
94261 ttttccatc  
94321 ttttccatc  
94381 ttttccatc  
94441 ttttccatc  
94501 ttttccatc  
94561 ttttccatc  
94621 tttt

47821 tctgatcatc ggcaatgggg attctacatt gcagaccctg gtcctttctg ccagccttca  
 47881 gaccttgttt acagacctcc tgaggataca cttttctcac ttactatgtt tctttgatg  
 47941 gcaacctata cacctctcaa taaattttgg tttacttagg agctgagggg cacagaaacc  
 48001 tagaaaccta gattcaattt gtgccgattt atgagacaca cgcacgacaa tcctggaaac  
 48061 tttccctgat ctcactttt aaattaaccc cttattttaa tattctgcag actcagcaca  
 48121 gagcagtgtt caatggggac atggtccctg atcagcctt tggccctctc catgttgct  
 48181 atcagaacac acggcccttg ttccggcaca acaatgcaaa ttacaagat ttatgagaag  
 48241 aaggaaatgt tcctatctgtt ggttattca ggcacagca tcactggctc taacaggctc  
 48301 catactgctc agttccagac gttcgacagc tggacttggg atcgtatgtt gaggcgtgtt  
 48361 ctgatttgc ttagtgcactc actcattcac tcattcagtc attcagctga aatgttccaa  
 48421 atgcttaata aacgcaagac aatgaggctg aagccaaagg gatgcaacac agagtaagac  
 48481 acagtccttgc gcctcaaaga acttatattc cactggaga caaaatgcct gtatggtacc  
 48541 atagagaagg catgaaacgc atgagccagc aaaccccttgc tggaaagcctg cacttgcttgc  
 48601 tgagctgtgt gactctgggt aatttatcgg atcctttat gcctcagttt cctcatcagc  
 48661 aaaatgggtg tagtcatacc cagttcaca gaggatgctg aggttaacagc attcaatcaa  
 48721 ctgctgtgaa atctctaagc accatataga tattcaagat tctttaaacac atcagaatgt  
 48781 cacaagagaa aggaacat atgtcgtgtt ttaacaagcg aaagactatg aaaaatcaga  
 48841 tacaaggacc ataaacccatc tggggatgtt aaaaaagat gtcctcactg cacgtttag  
 48901 gtattgttaac ccaattttctt gaaactcgcag aagaggacaa tgatgaggaa ggagaataga  
 48961 aagcaggagg aggaggagga ggaggagatg gctggccat taagtaaaca tgagaacagc  
 49021 cacatttagag aaacaagctg tatgcaggaa agagtcaag ttgaggactg gtataaagaa  
 49081 agagcttgc atccctgtc tttgagagaa tgaggtgaga ggactgctt aagccaggag  
 49141 ttcaagacca gactggagaa aaaagcaagc aagcccatc tctacaaaaaa aatagtcaaa  
 49201 attaaccacag cacaggagtg catgcttgc tgcctcagata cttggcaggc agaggcttgc  
 49261 ggatcgcttgc agccttgc tttggaggctg cagtatctc tgattgtacc actgcactgc  
 49321 agccttggca aaagagcaag acccttctt aagaaggaa aaaaaaaaaaa aaagatcgag  
 49381 agaaaaggct gaggctggcc acccttgc tctatatccc accaaactgt taacaccaat  
 49441 ggggacatag cacattgtcc aaatggcaga aatcccttaa agaaaagttt ttactttcag  
 49501 agagaaaaat aaatagaact gcatgagctc aaaagtcaact ggtgaaataa ttataaattgg  
 49561 atgtcatctg tctttttttt aagttctgtt atgaatatca cagaagaagc tcaaggttcc  
 49621 ctctgttaaag ggcctcccccc aacttcacgtg ctggccctgtt tgctccacca tgacacatga  
 49681 ttcccttggg ttgtccattc ttaatcaca atccctcctg agaggccac cacaggacat  
 49741 ggattcacac tagcaggatg atgacttttca taatccagga aggagagagg gacgcatttt  
 49801 cccacacttcc atggccacttgc ctttttttttccca accatggccgatc accatggatc  
 49861 cagggtgtgtt gggtggcgatg ttttttttttccca accatggccgatc accatggatc  
 49921 gaccccttgc gcaatgggtt gacccggatc ctttttttttccca accatggccgatc accatggatc  
 49981 tcttcttaatc aatagaatgt agctaaatgtt accatggccgatc ctttttttttccca accatggatc  
 50041 atggggctta aaaggccatg ccacatcttgc ctttttttttccca accatggccgatc accatggatc  
 50101 gggagaccatg atgacatattt gtggggcttgc ttttttttttccca accatggccgatc accatggatc  
 50161 tgaagaacttggggcccttgc ttttttttttccca accatggccgatc accatggatc  
 50221 cccttcgttgc aaggatttttttccca accatggccgatc accatggatc  
 50281 aaagacccttgc agcttgcatttgc ttttttttttccca accatggccgatc accatggatc  
 50341 aatggatc ttttttttttccca accatggccgatc accatggatc  
 50401 gcagataaccttgc ttttttttttccca accatggccgatc accatggatc  
 50461 gttatccatc aatggatc ttttttttttccca accatggccgatc accatggatc  
 50521 atatgcgaatc ttttttttttccca accatggccgatc accatggatc  
 50581 ctggcttgc ttttttttttccca accatggccgatc accatggatc  
 50641 tataatcatttgc ttttttttttccca accatggccgatc accatggatc  
 50701 ttttttttttccca accatggccgatc accatggatc  
 50761 catatcttgc ttttttttttccca accatggccgatc accatggatc  
 50821 catatcttgc ttttttttttccca accatggccgatc accatggatc  
 50881 ttttttttttccca accatggccgatc accatggatc  
 50941 actgcatttgc ttttttttttccca accatggccgatc accatggatc  
 51001 acatccatc ttttttttttccca accatggccgatc accatggatc  
 51061 tacatcttgc ttttttttttccca accatggccgatc accatggatc  
 51121 gatatttttgc ttttttttttccca accatggccgatc accatggatc  
 51181 gggggaaaatc ttttttttttccca accatggccgatc accatggatc  
 51241 ccccaatttgc ttttttttttccca accatggccgatc accatggatc  
 51301 tgcaagcttgc ttttttttttccca accatggccgatc accatggatc  
 51361 acatccatc ttttttttttccca accatggccgatc accatggatc  
 51421 ctggccatc ttttttttttccca accatggccgatc accatggatc  
 51481 ttttttttttccca accatggccgatc accatggatc  
 51541 gctgttaggg ttttttttttccca accatggccgatc accatggatc  
 51601 ggagggggggg ttttttttttccca accatggccgatc accatggatc  
 51661 acaagcttgc ttttttttttccca accatggccgatc accatggatc  
 51721 ctggccatc ttttttttttccca accatggccgatc accatggatc  
 51781 attcaatttgc ttttttttttccca accatggccgatc accatggatc  
 51841 gtggaaatgtt ttttttttttccca accatggccgatc accatggatc  
 51901 tgatgtgtt ttttttttttccca accatggccgatc accatggatc  
 51961 cacttcttgc ttttttttttccca accatggccgatc accatggatc  
 52021 ccataccggccatc ttttttttttccca accatggccgatc accatggatc  
 52081 ttatgtgtt ttttttttttccca accatggccgatc accatggatc  
 52141 gacaataacc ttttttttttccca accatggccgatc accatggatc  
 52201 ttttttttttccca accatggccgatc accatggatc  
 52261 gctgttgcag ttttttttttccca accatggccgatc accatggatc

52321 ctccctatgt tactatgtta cccaggctgg tcttgaactc ctgacaccta gtgatcc tac  
 52381 cacttcagac tcccaaagca ctgggcttac aggtgtgagc cactgtgtct ggccccaaag  
 52441 tattttaaag caagccccag acctcacatc attttcctc tgctgaatgt atatgtcaga  
 52501 tggtatttag aagaggcggg gttgctctgg tgctcaacag ctttctcat taataaaact  
 52561 actcaaagaa aaatgttagc aggagggagg agctctggaa ttaggagttc caattttggc  
 52621 tacaatctgc aattcctgtta gnatcttggg atactctgaa cctctgggtc tcagttcct  
 52681 taggttgggg aggataatga ctccctagacc tgtagggga actgaatgag acatgactt  
 52741 ctaaaaatcc tggcacagaa cctagtacag gctaaggggaa aggtgtgatg attgactata  
 52801 tgccttggg acacaggat acaacaccaa aaataaaaag aaggcaaaaaaaa aaaaattgt  
 52861 ggtgtcaact tgactgggg acacagtgc cagatatctg actaaccatttctgat  
 52921 gtgtgtctgt gagggtgatt ctacatgaga ctaaccctt aatcagtaga ctgagtaaaa  
 52981 ctgattggcc tgcctctgt ggggtggatct catctaattt gttgaaggcc cgaataaaac  
 53041 aaaagtctga gtaagaaaata attttctctc tttgcctgac tgccttcac ctgggatgct  
 53101 tgcctctgc ctggatgtt ggttctacc atggggccctt ctggatctcc agattgctgg  
 53161 ctacacttct tgggacttct gggcttccat aattgtgtga gccaattcgc cagttctaa  
 53221 aaatctctat ctgtccatct gtctagctat ctgtctgcct atctgtctct ctctctcc  
 53281 atccatccat ccatccatcc atccatccat ccatcttcca gatggaccct gactttagat  
 53341 ggttctgtt atgatgtttt gaaatgttga tggggggaa tggcaaggag aagaagtcca  
 53401 agggtttaaa tcctgtttt gccattcatg gctgtgtcgc ttggcaagt cccttaacc  
 53461 tctataagtt ttgattacct tatctctaaa agggggaaaa ctacatctac attttaggat  
 53521 tacataacaag aatgcaatgc agaaaaatgct gtc当地 aactggcctg agagtctcc  
 53581 ttacatagtt tccaatccag ctgaggctaa atggcaatttga caatagagaa atataattt  
 53641 tgcctactt atagggttgg aggactagag acgatattgc taactacatgat taatacagtt  
 53701 ccctgcacat aattggcaca cacataatta ataaagggtca gactgtgtgaag tttgttaat  
 53761 aatgatagat tataatcactt ttatttattt gacatgtgtc caatagaatt tggatata  
 53821 taggacctaataaaatgata tagttataaa ttaattattt attatataatttatttattt  
 53881 aatgataatt taaaatggcc cagtgcgtt ctgacttta gatggtagac accccatagg  
 53941 cagatggcag ggaacagtaa cactgtatggt gacgtatggt gtagaaatctt gtacacgtc  
 54001 atattgccta aatgttggtc ctaaccgtat ccgttctt cactcacccctc cagcccaacc  
 54061 tcctattctg catccccccag ttttgcctg gcccctgggtt taacaccctc gtc当地  
 54121 ggcttcttc tctggaggct gtgtttttat tgcatttgc ttggccactg tgacaggag  
 54181 cagaagccag gtcttgaatgtt cctcagccccca caactcttgc gggcgtaca gattagatta  
 54241 gtc当地ctt ctc当地tta ctgtcaactt ctgcctctg taatgttggc aggggtgtca  
 54301 ttaaggcctc acagccctgag gagatataatg acacaccagg gtc当地catttgc  
 54361 gc当地atgtt gcaatcttgc acaccctc gtaatttcc ct当地tacc tgacaattt  
 54421 aacagcaact ctgtcccttgc tcatgtcaac cagcttagcc cccttactct gactcctaat  
 54481 gaggagggtgc agtgccttgc tcccatccctg aaataacaac actggctgt ggc当地atgt  
 54541 ttgttgggtt tggatgttttctt ctgaaatggt gtcttgcctt cc当地ccaggc tagagtgc  
 54601 tggccatc tggctcaact gcaaccgtt gtc当地ccagggtt tcaatgttgc  
 54661 agc当地ccaa gtagtgggaa taacagacat gcaaccatccat gcttgc当地aa  
 54721 ttggatgttttctt ttagtagaga tgc当地tttca ccatgttgc caggctggc  
 54781 gacctcaggat gaccacccca ccttgc当地tcc ccaatgttgc gggattacag  
 54841 ccacaccctt ccccttttcc tcttagactt cacttccat gtc当地tttca  
 54901 ctccctggcc gaaaatgggg ctggaaatgtt gtttgggat gacttgc当地  
 54961 gc当地tggaga ctacacacccat gaccctggag ccacagagac tccagctcc  
 55021 gagttgttgc caccggactt taccttccctc atctgtaaaa tggcaatgat  
 55081 ccccccacaca tggctcttgc atgtcatctt cgttgc当地tcc ttatcacaga  
 55141 tggacatgtt gcaatataaca tctgtcttca ctgtcttgc ggttgc当地  
 55201 catgc当地tta cccttcaact ttagtgc当地tca gtactttgc  
 55261 tgatgttgc当地tcc cccacagaaa ggc当地cttgc当地tgg  
 55321 agagaaacca gggatcaaag ctctctgggtt cc当地tgc当地tcc  
 55381 ggaaaggcag acggggagga aataatctaa accccgttgc  
 55441 aagaaacata ttcaaaaaat gtaatagctt ggtgtccggc  
 55501 ataccactt cacttacaga gaaaggggaga tggaggcaga  
 55561 aattgttcaaca cagctggtga ggggggtggc  
 55621 cctc当地actt gtgtcttgc当地tgc当地tgg  
 55681 cccaaatccat gcaacttgc aaggcaatgtt  
 55741 taatgttgc当地tcc atcttgc当地tgc当地tgg  
 55801 ttcatagcc agatgttgc当地tcc  
 55861 gagaaggccat tggctcttca gaggcagg  
 55921 cgagcagccat cc当地tacca  
 55981 aagtataatgtt ctgc当地tgc当地tgg  
 56041 ctgttgc当地tcc tggatgttgc当地tcc  
 56101 acgtatgttgc当地tcc  
 56161 tcccaatgtt ctgttgc当地tcc  
 56221 gggatgttgc当地tcc  
 56281 ttgacttccat cagctgggg  
 56341 aaatgttccat acaaaatgtt  
 56401 gctatggggat tggctcttgc当地tcc  
 56461 atggggatgttgc当地tcc  
 56521 ggcttcttgc当地tcc  
 56581 cccagaaacccat  
 56641 tggctcttgc当地tcc  
 56701 agatgttccat  
 56761 gcccaccatc



61321 actcagaacc aacgaatgtg actttatttgc  
61381 taaggaatga gatgagatca tactcaatta  
61441 ttgttaagaga cagaaaggac acaaagccag  
61501 gattagaatg tggtgttcc aagccaaggg  
61561 ccaggagaca ggcatggcat ggtttctccc  
61621 aacgccttga tttggactt ccggccacca  
61681 taccggatgtt gcgataattt gttatggcag  
61741 ggttataaaa tgcaaggcac ttccctgaag  
61801 ttgtcattat atctcaagga aggcccacac  
61861 aaaaccaccc aaattttctt agtggcata  
61921 caagaagttc tcagatcatc agtaagaaag  
61981 ggaatactga gatgtctcca aacaatactc  
62041 tggcgttattc agggcaccac ttccactgtt  
62101 actttgggtc acacatggaa aggacattct  
62161 aacttagcag tgcattcatc tggttctgt  
62221 gggcttctt tatgtctcca gcaaggcagg  
62281 tagatgtcga ggcattcatc aggtgctcac  
62341 acagaggtaa atagtaagaa caatgcccaga  
62401 taccatcccc atttacaga tggtgatata  
62461 agtgaataca taaataagta aataatgagt  
62521 gttcaagtct cactcaaaga agactgagtg  
62581 ttaaccatca gagatattgc caagctttgt  
62641 cttccactgt gctggatg caggcaggc  
62701 gtttcttggc tttgcccagg aaagaattca  
62761 tttaaggggc agtgcataac aggagcagag  
62821 acatgcagag taccacagt gcaattccg  
62881 ttttaattat atgcaaaatc agtggtgat  
62941 acttccaggta tgggggtca ttgtcatgga  
63001 gaaattgtaa actgacatga cacactggcg  
63061 tatccctgtt tgactgtc ctaattttgg  
63121 agtcttgcctt ccagaatcaa gtcccccac  
63181 ggcctgtca gccccggcatttccatc  
63241 gaagtgaact gaacagcagt ggcacactga  
63301 ttctgtggaa tgaacacctg agtactatgg  
63361 atcgtttcca ttaattttc tcaacaagcc  
63421 agagagatta agtggcttac cctggaacat  
63481 tttcctgact ctgctttaa gccgtacact  
63541 gctaacaatg ttctgccaatttcttact  
63601 tactggctcc aattcagaaa agacgaaact  
63661 caagctgtt acatggtaga ggcacactc  
63721 cttagtccatc tgacgctcg acctaccagg  
63781 agtagcttagg actacaggca tgtaaccaca  
63841 gattgggtct cgctgtttg gccagactga  
63901 tgccgtggcc tcccgaaatgt ctgggattac  
63961 ctgggctcctg ctgtcttag ggcttctac  
64021 gtagaacacgc tcaaccaacc ctaatcaaca  
64081 ttgtgtggc gggctgggc aggactgtga  
64141 tgatgtgcag cccaggctc cttcccgagg  
64201 cagggagtagc ctgaacacag agactacatt  
64261 caccatca ctagtcaagg caggttata  
64321 tagctctgat gggccctttt agttcataa  
64381 tcagtcagct tcctcccttg cccactccat  
64441 ttccaaggca cgtgttcttca aacaacaatc  
64501 aatgagatgg ttctcattt ctaaagaaaa  
64561 atcagaaaaac ttctcattt ggtactgtga  
64621 agggtaggaga ggcgcataatc caggggaaaca  
64681 aattttagga ggaagccaaag cgaagacagg  
64741 ccaataccca ggagtacagg acttaacagg  
64801 tagtgcgtgc tccaaggcag aggtttgtt  
64861 ttggcaatt tgaagccaaag ttccagccaaag  
64921 cacatccaga gaagccaaact ctcccgatag  
64981 aagatattag ctcttttaagg aggccaaatgc  
65041 gagaatgccc ttctgggttc ttctaaaatt  
65101 atcaagtcta cacaacttgc ccaggcttac  
65161 agcttcccta ttctttgtga atcagactca  
65221 aaaaaaaaaaa aagaatagca ctaatgttta  
65281 caactttca ttcccttagt ccaccaggaa  
65341 tttatccatc caaacgtact tcagactgt  
65401 caagtgcaggat cttgggttggg caatagctca  
65461 ctaataataa cttactctgg gacttgcata  
65521 ttgttaacca ggtgtatgaaa aattttggaa  
65581 gattgcata acttttgcactaaatgtat  
65641 cccaaatcaga gccaacccca gtttcttcac  
65701 cattttaaag tgacataaaaa gttgcctgaa  
65761 tttgtacacca cttacatctgtt atttactatt





74821 actccacatt tgccacattt gaatcaggtt ttagccactg tcgttaatta gaatccatgt  
 74881 agaagcaaaa acattaaaaa taaacccggg cccttgatgt tatacgctc atttttac  
 74941 atggcctcat gattcagcgta ttaatggacc taaaacaat cataaaacat caaggcctgt  
 75001 gttcaagctt agaagaacaa gaatagttt acttcgtctc tggctctt gtttagatt  
 75061 cactgggtgt cactggcattc tggtaaacact ttaaagtatt ttgacattgt ggtatgagat  
 75121 gttgttaatc tggccctgc ctgacctca ctggccagtg gcatgttgcg actcagtgg  
 75181 aaattcatga aaaacacgaa ggtatgtactc agtgacatag attaggacac aataaaccta  
 75241 aataatggcc ataatgtgc gattccaat caatacatgg acccacaaaa gacttattga  
 75301 gcatccatc tgccacaggc acttgggtga tgcctgcaca aattacagat ttatggca  
 75361 taatcctcag cacaatgtg ggtcagcat tagtgcctt catttcatg atagcatgc  
 75421 taagacccag tcatgtggct agtaagaggc agaggtagga ttcaaaaagca ggttctga  
 75481 ccccttgcctt aataccattc ttttccaaat atccactgc aggacacgaa ctgttgc  
 75541 atgtggcaga ctgtatctt caaaagtggc catggcagta tgcctcactt cacattctt  
 75601 tccagaagag gtgggtctt tttccctca ctgcaacactt ggtgggggtg ggtctgac  
 75661 cctaaaaaat agaatgcagg aaaacctagg gctatgtac ttctatggaa gcccataaaa  
 75721 ggcaatgcag catctgtctt gttcttctt ttgggacgct cacccttggg acccagccgc  
 75781 catattgtga ggaagctcag ggtacatggc gacaatgtgt aggtgttcca gccaatagac  
 75841 cctccagcaa gggcccccacc caccatgtcag catcattctt cagactcatg agtgaacaac  
 75901 cttcagatga ttcagatccc tggcttcaat gctgttcag ctgtatgtt gacagagaca  
 75961 agctgttctt gagacccctg ctgaaaactac agattctga aaaaaataaa tttttgtt  
 76021 ttaagccact aaatttgggg gtgggttggt atgcaacaat ttgtactggg atatagtc  
 76081 actttaatga aactctaaag aatagggaaa tgcttcattt ccctcatcaa tgatattcc  
 76141 atggccagaa aatgggttctt catgaaagaa atcctgagag gaaaaagttt aaggtctt  
 76201 ttgttagaaaaa acaccaagaa ttggaaaggc ttattatatc agggattaa gtgaaacatt  
 76261 tggaaagaaaaa gaaaacacgac agcctaataatg atataccac aagtttagat ttatggatt  
 76321 atatatttat tgcttgcact gctaagatct atctagagcc tcaagttggct ttggattat  
 76381 tcattatttg gcttggaaat atttcttgc ttaagggat cctccactt tgcctcgaga  
 76441 ttatagggttgcattttttttt ttattccctt atgttcaaaa gatgagaaaaa atgttgc  
 76501 catgctagaa agaagaccca acaaggcaga aaaaaactct taatcttctt atattcg  
 76561 atcttggcac tgccttgcactt agttggaga ggaactgaaa acagaaacaa aaacaaaaca  
 76621 aaaaactggat atctatggag atatagccgt ataaagagtt aaaggaagtt ggcaaaagagt  
 76681 gacagtactt ttttttgcactt gttttttttt ttcaacccaaa taagaagctg tttaagtctt  
 76741 aacagtattt aatctaaaaa tccaaatgtt atccactt ttcgtatcta cttcaagat  
 76801 gcctcaatc tatgtgttag gggggggggc tgcgttcacg cttgtatcc cagcactt  
 76861 ggaggcttag gggggggggat cacatgtca gggatgtcgg accatcttcg ataaactgt  
 76921 gaaacccttgc ctctacggaa aacatgaaaaa atacaacaa tacaaaaaaaa aaaattagcc  
 76981 aggcgaggtt ggggggtgcct gtgtccctt gttttttttt ttcaacccaaa taagaagctg  
 77041 atgaacccgg gggggggggc ttgcgttag cggagatccctt gagatggcgc cactgcact  
 77101 gggccctgggc gacaggggtt gactccgtt cttttttttt ttcaacccaaa taatgggg  
 77161 gatttgatttccatggggca gttttttttt ttcaacccaaa taatggggca ggggggggg  
 77221 ttcttggta aatattttttt ggccttccat aatattttttt ttcaacccaaa taatgggg  
 77281 atatttttgtt agttagatccat tttttttttt ttcaacccaaa taatggggca ggggggg  
 77341 atttttttaatggggca aatattttttt ttcaacccaaa taatggggca ggggggggg  
 77401 caaataaaaaa tggaaaacttc cccaaatataca atggaaacca tccgtacaca ttggtag  
 77461 tatttcaatc ttttccatggaaat ttgttagttt ggggggggggggggggggggggggg  
 77521 aaaaacccgtt atactgtcat ggccttccat aatattttttt ttcaacccaaa taatgggg  
 77581 aggattttttt tttttttttt ttcaacccaaa taatggggca ggggggggggggggggggg  
 77641 aggttaactttt tttttttttt ttcaacccaaa taatggggca ggggggggggggggggg  
 77701 aatttagaaaaa aaaaacacgaa ttggaaatgtt atgtttaatgggggggggggggggg  
 77761 actcaaaaggc tttttttttt ttcaacccaaa taatggggca ggggggggggggggggg  
 77821 gaaatccatc ccagg  
 77881 cagaacatcatc tttttttttt ttcaacccaaa taatggggca ggggggggggggggggg  
 77941 gtctttttttt atcattttttt ttcaacccaaa taatggggca ggggggggggggggggg  
 78001 cttttttttt accttccatggggca gttttttttt ttcaacccaaa taatggggca  
 78061 gagtttttttccatggggca gttttttttt ttcaacccaaa taatggggca ggggggggg  
 78121 accagccggc tttttttttt ttcaacccaaa taatggggca ggggggggggggggggg  
 78181 ggg  
 78241 attaaaaaaaactt tttttttttt ttcaacccaaa taatggggca ggggggggggggggg  
 78301 tttttttttt ttcaacccaaa taatggggca ggggggggggggggggggggggggggg  
 78361 ggg  
 78421 aagaaggagg gggcccttccatggggca gttttttttt ttcaacccaaa taatgggg  
 78481 accccacacac acacccatggggca gttttttttt ttcaacccaaa taatggggca  
 78541 atttttttttt tttttttttt ttcaacccaaa taatggggca ggggggggggggggggg  
 78601 ttccatggggca tttttttttt ttcaacccaaa taatggggca ggggggggggggggg  
 78661 tttttttttt ttcaacccaaa taatggggca ggggggggggggggggggggggggg  
 78721 gttttttttt ttcaacccaaa taatggggca ggggggggggggggggggggggggg  
 78781 tttttttttt ttcaacccaaa taatggggca ggggggggggggggggggggggggg  
 78841 aggcaggagg tttttttttt ttcaacccaaa taatggggca ggggggggggggggg  
 78901 tttttttttt ttcaacccaaa taatggggca ggggggggggggggggggggggg  
 78961 tttttttttt ttcaacccaaa taatggggca ggggggggggggggggggggggg  
 79021 catgagttttt tttttttttt ttcaacccaaa taatggggca ggggggggggggggg  
 79081 ctggccaaat tttttttttt ttcaacccaaa taatggggca ggggggggggggggg  
 79141 ggagagggaaa aaaaaaaaaaa tttttttttt ttcaacccaaa taatggggca  
 79201 aacccatggggca gttttttttt ttcaacccaaa taatggggca ggggggggggggg  
 79261 tggatccatggggca aagacttataat tttttttttt ttcaacccaaa taatggggca

79321 ttttttaaaa ataaagcacc tactagatac tcttgcacct actggccaca gtacaaggca  
79381 ccatgaattc catggcaggg ctggtgatga aaatcattt acctttctt ccactcact  
79441 gttcattttt ctgtttttt tttttttttt tttttttttt tttttttttt tttttttttt  
79501 ttttttttca gatgaagtct tgctctgtga cccaggctgg agtcaatgg catcatctt  
79561 tctcaactgca acctctccct cctgggttca agcaatttctc ctgttccggc ctcccatgtt  
79621 gctgggattt cagggccccca ccaccacacc tggctcaactt ctcttattttt agtagagacg  
79681 gggcttcaacc atatttccccca ggctgtatcc gaactcttga cctcaagttt tctgcctgc  
79741 ttggcctctc aaagtgtctt gattacaggc atgaggcagc gtggccagcc attcaaccat  
79801 taatgcatta ctttagtca ctaactatattt cacaatattt tatttaattt tagttaaggaa  
79861 caaaactagt gactaagttt tggggaaaat ggttggctt tgcctcaac tatttaataat  
79921 ttaagttctg gtaaaaatttcc ataaaatttcc agattaat tattttttt tttttttt  
79981 cataaaactac tggttattttt aatacacttgc cttcctcgcc atttatttctt tcaacctac  
80041 ctgttcaattt ccattcacag acctaccctc tttctcttgc taacaatttta tttcccttcc  
80101 tttgtccttat agcattgtctt gggaaaatggaa acaccggta aatactttaa cacagagcc  
80161 gtgcacagaaaa gcttgcaccc cattcagttaa acaccaggca aaaaaaaaaa aaaaaaaaaac  
80221 ctaaacaaac aaaaataaaaa aaaaacttcc tttttttt tttttttt tttttttt  
80281 aaaagcagct ctgatcttgc agtgagatc acgcacaggc acatttcttgc tcaacttcc  
80341 aatgacaataa atgctaataa taataaccagg cagagccagg aagagccacc atggtacacc  
80401 agcctctggg ataggccctt tccacacatc tttttttt tttttttt tttttttt  
80461 ttttcttacc ccattttgtt gaaaaaaatcatc tttttttt tttttttt tttttttt  
80521 acaaaccagg ttaatttttag atcaggacaa tttttttt tttttttt tttttttt  
80581 ttcaagttttaa attgtatatac ttacacagct tttttttt tttttttt tttttttt  
80641 ttgtcttggaa ctgaaatgtc atcaatccatc tttttttt tttttttt tttttttt  
80701 ggggggtcactgcttggaa gaggtatccaa ctaactccatc tttttttt tttttttt  
80761 ataaacttcat tcagaaacaa agttaaggag tttttttt tttttttt tttttttt  
80821 aaatttgcocc agtaaggact ttggctgtcat tttttttt tttttttt tttttttt  
80881 ggaagctaaa atagcaaggg tttttttt tttttttt tttttttt tttttttt  
80941 gttggagtgc ggtgacatga tttttttt tttttttt tttttttt tttttttt  
81001 ctttccctgc tcagccctt tttttttt tttttttt tttttttt tttttttt  
81061 atttttgtat tttttttt tttttttt tttttttt tttttttt tttttttt  
81121 ccaaccctca gggatctacc tttttttt tttttttt tttttttt tttttttt  
81181 caccatgcccc accacaaaaaa tttttttt tttttttt tttttttt tttttttt  
81241 accacttggat atgatgtatca tttttttt tttttttt tttttttt tttttttt  
81301 agattgcacc actgcacttcc tttttttt tttttttt tttttttt tttttttt  
81361 aaaagaggta gagagagaat tttttttt tttttttt tttttttt tttttttt  
81421 aaactattac atgggggttat tttttttt tttttttt tttttttt tttttttt  
81481 tccatttggat agtttggatgc tttttttt tttttttt tttttttt tttttttt  
81541 aactatcaga ctgagtttttgc tttttttt tttttttt tttttttt tttttttt  
81601 ctatctcaag agaagtttgc tttttttt tttttttt tttttttt tttttttt  
81661 ctttagctaa gaatgtccat tttttttt tttttttt tttttttt tttttttt  
81721 agtcagggaaac ccccttaaaaat tttttttt tttttttt tttttttt tttttttt  
81781 gccgtcagct gccactgcaat tttttttt tttttttt tttttttt tttttttt  
81841 tggggacaga ttctgcataat tttttttt tttttttt tttttttt tttttttt  
81901 ccccccgttcc tagaccccaat tttttttt tttttttt tttttttt tttttttt  
81961 tggggccattt ttcataagccat tttttttt tttttttt tttttttt tttttttt  
82021 atataacgc agctggtcac tttttttt tttttttt tttttttt tttttttt  
82081 gtgttaaaaacacacggat tttttttt tttttttt tttttttt tttttttt tttttttt  
82141 ctgtggctga aaaaacttccat tttttttt tttttttt tttttttt tttttttt  
82201 attaaaatcgt catctaccgc tttttttt tttttttt tttttttt tttttttt  
82261 ttgcattgaaa gatattctaa tttttttt tttttttt tttttttt tttttttt  
82321 aaacgtatca ttcagagcc tttttttt tttttttt tttttttt tttttttt  
82381 tccttccctt ctgcccattt tttttttt tttttttt tttttttt tttttttt  
82441 gacctgcaat gccagacggcg tttttttt tttttttt tttttttt tttttttt  
82501 tcaggaaaaat gtagtcttccat tttttttt tttttttt tttttttt tttttttt  
82561 ctaatatgca tttttttt tttttttt tttttttt tttttttt tttttttt  
82621 aaagatattctt aaaaattttt tttttttt tttttttt tttttttt tttttttt  
82681 gtgtactttt ttttagagaaat tttttttt tttttttt tttttttt tttttttt  
82741 agaaaaggta gtagccaaat tttttttt tttttttt tttttttt tttttttt  
82801 aaagccatgt gaccttggcc tttttttt tttttttt tttttttt tttttttt  
82861 cataaggaga gattcggat tttttttt tttttttt tttttttt tttttttt  
82921 tttaaaacaaa cttttttt tttttttt tttttttt tttttttt tttttttt  
82981 ttccatagaaa atttttttt tttttttt tttttttt tttttttt tttttttt  
83041 agcatgtttt tttttttt tttttttt tttttttt tttttttt tttttttt  
83101 atctacccttgc gggttttcat tttttttt tttttttt tttttttt tttttttt  
83161 aaagagacag caagacacaa tttttttt tttttttt tttttttt tttttttt  
83221 tcttttagaaa ggcagat tttttttt tttttttt tttttttt tttttttt  
83281 acaaatttgcacca ggaaccataa tttttttt tttttttt tttttttt tttttttt  
83341 tgacaaaatg gattgacaaat tttttttt tttttttt tttttttt tttttttt  
83401 ccggaaaaaggc attttgcacttgc tttttttt tttttttt tttttttt tttttttt  
83461 gcaccacccgg ccccaacaaa atgcccattttt tttttttt tttttttt tttttttt  
83521 taattttccat gggggagaaaaa ggacacttgc tttttttt tttttttt tttttttt  
83581 atctgtatgtt gaaatcttgc tttttttt tttttttt tttttttt tttttttt  
83641 tactttatgtt tttttttt tttttttt tttttttt tttttttt tttttttt  
83701 tttttttt tttttttt tttttttt tttttttt tttttttt tttttttt  
83761 aatataatatgtt tttttttt tttttttt tttttttt tttttttt tttttttt



88321 aaagacccca tctccctaaca aatgaatgct ggggtgacac agacattctg accacagcaa  
88381 gatttgaatc tagcctgctg taacccaaag cctgtgttac taagaacttt gctatatggc  
88441 atgggtcctt ctgagcccta cctgcccac atccttctt atatcaaaaa tttcaacttt  
88501 tttttcaaa tattttatc tttgttagaga tgaggaggtc ttgaactctt gccctcaat  
88561 gattctcctg cctcggccctc ccaacgtgt gggattacag acgtgagccca ccttgcgg  
88621 caaaaaattt tcattgttcc taacctggat tcctcttcaa accttcttag gcaatgtt  
88681 tcccactgca aggatgaggc gcatttgcac cattttgtaa tgtaattata agttatttcc  
88741 ataatggctg aatagtctg aaaaaaagt tttttgtgt gtgtctatgt ggcacattga  
88801 ataattatc aacaataaaag gttacctt atgtactaca gctgtgttt atgctaataa  
88861 ccagaatcat ttaatcattt tgagctaatg acctgaaaaa ttcatattaa aaaatgaagc  
88921 catatctggg tctctggaaa gcaaaactgg ataaaaaattt agggaaagtca atgttcaatt  
88981 tgtattttt agtggagagc agccgtatag tctgtcagt tcacatccaa gtttgagcat  
89041 ttaaacagag gcagggccct ggcattgtg ctcacccctg gaatcccacg actctggg  
89101 gctgaggccag gcagatctcc tgagcccaag agttccagac cagccagtgc aacatggca  
89161 aacaccgtct ctacaaaaaa atataaaaat tagctggca ttgtgttccc agttacttgg  
89221 gaggctgagg tgtgaagatc acttgatcc atgagacagc agttgcagt acctgagatt  
89281 gcatcactt actccagccct gagcaacaga gtgagggct ttctcaaaaa atagattaga  
89341 tataagatatac gatataataga taagatgaaa gaaagaaagg gggcagcggg ggcaaggaaa  
89401 ttccgcacatc taagggactt aatggatatac cttgagcacc aacgaaataa agagatttcc  
89461 tattgcattt ccatggttat caaggttttt gtgtgcgtt ttccttctgt ctgtacacga  
89521 ctctgtcctt ctatgtggaa ttctgttact gcttaatccc cggggtgagc agagagcaga  
89581 gctgtggaca atcagagctc cctgctggca atcttggta ggtatcttggc cttccttggc  
89641 ttcccaaccc tgaaccaactc tttgtttttt ctaaccctca tttaagatt tctacaggt  
89701 agatgcctct atagacttgt atagtaatgt cttatcatttgc tttttttttt aggtatcc  
89761 cctcttttct tactaaaata attataatc tttttttttt acagagcaat gaatcaaaaat  
89821 caatccaaacg cagtgtgtg tgcataattt gtattaaatg gcttcaagtt ttacgaacc  
89881 ctttatccat gcccctgtcc gtgcagactt gcagcacctt ttcagttgtt aagtggaca  
89941 tacttcctca ccctggagca cgaccatatg atttgccttgc gcccagaatg aagaagcaat  
90001 aggaagccat ctccgagctt ttatgtttc tgcctgtttt ttctgtttt taccttaac  
90061 catgaaaagg atacatgtgg gaagtagagg agagagatcc aaggtggggc tgggttactc  
90121 cagccaaatc cagccatagat cagcccatcc ccagccccat tggccagactc gtgagaaaga  
90181 tgagcagacg aatgcagggg agcttagcca aggtcaccc taaaacccagag atggcgagaa  
90241 tcgaatgtt attgctgcgg gccactgagg tcaatgtgtt gtttgcgtt cagcaagc  
90301 gcggaatga cttaagggtt gctcaagggtt aaataaggga atgtccagta ctgacttctg  
90361 agctgactat gggatgtgtt gctcaagggtt aaataaggga atgtccagta ctgacttctg  
90421 aaagaaattt cttcccttaa aaaaacaaaaa aacaataaga aaaacttggt aagagaaggc  
90481 ctctttatc tatttttttgc ttctgtttt tttttttttt ttgtgtttt ttttttttt  
90541 aaaaaattt cactgtcgcc caggctgcag tgcagttggc cgtatcttggc tcactgcaac  
90601 ctctgcctct ctgggtcaag tgattctcc gcctcagcc tccaagtagc tgcaattata  
90661 ggcaggccccc accaaatccc agctaattttt ttgcattttt agtagagacg gggtttcatc  
90721 atgttggcca ggctgtctc gaacttctg ctcaggttga tccacccatc tcattatccc  
90781 aaaaatgtgg gattacatgtt atgagccacc gcaccccgcc cttctgtttt cctcagattc  
90841 actaacttca gatggccttc gaaaggatatac ttcttagagc agtggaaaccc atctggggaa  
90901 cacaagagaa aggccaagac cacgacaggg caaatgtgtt acatcaagga gtcactc  
90961 cagccctggaa acagtctctt ccaaattatg ttccaggaga tgaatgtatgt ctccactg  
91021 catgccatc tcagatcgcc tgctccttgc tgctgaatgc ttcttaatac ataaaccc  
91081 gactggccgtt cagttccatg ccacaaacacc cagcacatca ccagacacac agggaaaccc  
91141 agtgccttggt ggatcgagg gagggtgtt gtttgcattt catgagttca tgaggcc  
91201 ctgtcccccggc accttacgc gatacggcc acatccatg ggtgttccgg aaagactga  
91261 gtcacagatg tatttttttgc actgacccctt aaccccttc ttcccttgc ttcccttgc  
91321 gcccctgtgtt ggaggcttgc gatgaagaaac aacgaagatt ttctttttt tcaactttt  
91381 tttttttttt ttttttttgc acagggtctt gatctgttcc acagggttggc tgcaatgg  
91441 gtaatcttgg ctcaactgaa cttccatctc ctgggtcttgc gtgttcc caccctc  
91501 tcctgagtag ctgggaccac aagcaagcac caaaacggcc ggttaatttt tttttttt  
91561 gtagaaatgtt ggtttccatc ttgttggccat gtttgcattt aaccccttaggc ttaagtgt  
91621 ctccccatc tgcctcccaat agtgcatttttgc ttacaggat gatctgttgc gcccagg  
91681 cagttagatg ttacttagat tacttttttgc tttttttttt tttttttttt  
91741 aacctttgtt ctgtgttttgc atgttccatc gatccatgc tttttttttt ttttttttt  
91801 gcccctgtgtt ggaggcttgc gatgaagaaac aacgaagatt ttctttttt tcaactttt  
91861 gttgtccacg gtatgtatgc ttgttgcattt ttgggttgc tttttttttt ttttttttt  
91921 ctctgtactt ttcttagagga gatttaaatgtt cactgtgttgc agactaaaaaa taataatgt  
91981 gagctggccca cgggtggctca cacctgtat cccagactt tggggggctg ggggggg  
92041 atcaacttgc gtcaggatgtt tgagagcagc ctggccaaaca tttttttttt tttttttt  
92101 tgaaaatatac gaaatcactg aggctgtctg gctgtgcgc tttttttttt tttttttt  
92161 ggctgaggca ggacaaatcactg ctggacccatc gaaggagggtt gatctgttgc  
92221 cactgcactc cagccccggc aacagacggc gaccccttgc caaaaaaaa aaaaaattt  
92281 aaaaaaaaata ataaatcttgc aacaataaca gtactcgatt tttaggttata aatgttcc  
92341 atgtgcacgg ttttttttttgc agagcccttac atgatataataa tttaatcttgc ttgtgtt  
92401 aataaggaaa gaacaactat ttgttgcattt ttatagtcg gggaaactgag gcttggag  
92461 gtgaagtaac ttgtccaaatc tgacacaataa agtggccatc ttgggttgc tttttttt  
92521 tgtctgatc agaaggccaa gatccatc acccttgc tttttttttt tttttttt  
92581 aaaaatatac ctttgcacgg ttttttttttgc aactggaaa tgatgttttgc tttttttt  
92641 tatgtcatgg ttgttggggat ctggccaaacc ttgttgcataa agtggccatc tttttttt  
92701 aaaaatgttttttgc ggg  
92761 aaaaatgttttttgc ggg

11

the above report in format

## **THE MOLECULAR BASIS OF BREAST CARCINOGENESIS**

April Charpentier, Ph.D. and C. Marcelo Aldaz, M.D.

Department of Carcinogenesis, The University of Texas M.D. Cancer Center,  
Science Park - Research Division, Smithville, Texas 78957

Tele: (512) 237-9530

Fax: (512) 237-2475

Acknowledgment: The authors wish to thank contributing colleagues Andrzej Bednarek, Feng Jiang, Kathleen Hawkins, Kendra Laflin; Michelle Gardiner for her secretarial assistance and Chris Yone for art work. This work was supported by Grants DAMD 17-94-J-4078 and DAMD 17-96-1-6252 from the U.S. Army Breast Cancer Program; NIH Grant R01 CA59967; and Susan G. Komen Breast Cancer Foundation.

## INTRODUCTION

Breast cancer is the most common malignancy affecting women today. This disease has reached epidemic proportions in the industrialized world afflicting as many as one in eight women (1), and causing approximately 45,000 deaths per year (2,3). In response to this major public health problem, research funding is being used to identify key steps in breast carcinogenesis with the goal of developing effective means for preventing, diagnosing and treating this devastating disease.

Overwhelming evidence has accumulated indicating that breast cancer is a genetically-based disease in which spontaneous mutation and/or hereditary genetic predisposition play primary roles. Environmental and epigenetic influences are also important, however their contribution to breast carcinogenesis is, at present, not well understood. The potential for developing breast cancer is most likely determined by the specific genetic makeup of an individual woman, however, this potential only becomes a threat by the interaction with specific endogenous as well as, perhaps, exogenous factors (Figure 1). For example, an important endogenous risk factor would include the extent of exposure to a woman's own ovarian hormones (4). Early age at menarche and nulliparity have been linked to an increased risk for developing breast cancer. It is likely that the extent of endogenous hormone exposure is genetically pre-determined or at least influenced by genetic factors. Examples of exogenous factors would include lifestyle choices such as, diet, alcohol intake and cigarette smoking (Figure 1). Physical environmental causes, such as ionizing radiation, have been shown to increase the likelihood of breast cancer development (5,6). Also, although

the topic is controversial and less understood, we should mention the potential role of environmental pollutants and hormone disrupters (7) (8). Taken together, it is then the combination of the genetic constitution, plus the influence of multiple endogenous and exogenous factors which, ultimately, will determine the overall risk of any particular woman for developing breast cancer.

Numerous mutated genes have been shown to be linked to breast cancer development although the assumption is that there are many more important breast cancer genes yet undiscovered. This overview focuses on the currently identified genes as well as genetic aberrations which may lead to the identification of as yet unknown genes key to breast carcinogenesis. In addition, other factors which also exercise significant influence in breast carcinogenesis, such as the hormonal regulation of cell proliferation, are discussed.

## **HISTOPATHOLOGY**

### *Normal Breast Tissue*

In order to correlate the genetics of breast cancer to the clinical manifestations, an overview of the histology of both normal and cancerous breast tissue is required. It should be mentioned, however, that it is beyond the scope of this chapter to perform a detailed analysis of breast tumor pathology.

The mammary glands are derived from modified sweat glands and basically represent downgrowths of the epidermis. In the adult woman the mammary gland is composed of approximately 15-20 lobes of branched

tubuloalveolar glands. Dense fibrous connective tissue separates the lobes. Each lobe in turn is subdivided into multiple lobules. Mammary lobules are located at the deepest end of the duct system. These lobules form clusters identified as blind-ending terminal ductules, or acini. These structures are embedded into a loose connective tissue rich in capillaries. Dense fibrocollagenous support mixed with abundant adipose tissue fills the interlobular spaces. The main collecting ducts (i.e. the lactiferous ducts) are lined by stratified squamous epithelium near their opening onto the nipple. A short distance from the surface each lactiferous duct presents a dilated portion known as the lactiferous sinus where the epithelial lining shows a transition to a two layer cuboidal epithelium. From there and throughout the rest of the duct system and acini the epithelium consists of one layer of luminal cuboidal cells and a basal layer of myoepithelial cells (Figure 2).

The development and differentiation of the mammary gland is hormonally regulated. The ovarian hormones estrogen and progesterone control breast development, especially during puberty. Full or complete differentiation of the mammary gland takes place during pregnancy and lactation, a time when the hormone prolactin plays a fundamental role. The explosive growth that the mammary epithelium undergoes during pregnancy causes the mammary tree to branch dramatically increasing the number of acini. This level of differentiation constitutes what Russo and Russo have described as lobule type 3 (9).

Wellings has suggested that the majority of breast carcinomas originate in what is known as the terminal ductal-lobular unit (TDLU) (10), also known as lobule type 1 (9). The TDLU has only 6-10 terminal ductules/lobule and is equivalent to the less differentiated state of

mammary gland development found predominantly in the breast of the nulliparous women (9).

### *Invasive Carcinoma*

The most frequently observed invasive breast carcinoma is the infiltrating (invasive) ductal carcinoma (IDCA). IDCA represents approximately 75-80% of the total invasive breast cancer cases (11). Most invasive ductal carcinomas display the typical phenotype of well to poorly differentiated adenocarcinomas. Another type of invasive breast carcinoma, which accounts for approximately 10-15% of the total invasive breast cancer cases, is the infiltrating lobular carcinoma (ILCA) (Rosen, 1979). ILCA has a very distinctive infiltrating growth pattern, characterized by the pathognomonic presence of isolated cells or cord of cells (i.e. Indian files pattern) (11). This tumor type also presents a different clinical and metastatic pattern than the invasive ductal type (13). For example, patients with invasive lobular carcinoma have been reported to have a higher risk of developing multifocal and contralateral breast cancer than patients with invasive ductal carcinoma (14). It is important to remember, that the designation of ductal and lobular carcinomas does not imply that ductal carcinomas originate exclusively in ducts and lobular carcinomas in lobules. According to Wellings, both tumor types, ductal and lobular, appear to originate in the TDLU (10).

### *Preinvasive and Hyperplastic Lesions*

In an attempt to clearly identify stages of breast cancer development, standardized terminology is used to refer to important changes in the morphology of the breast epithelium which have been noted by pathologists. This is shown schematically in Figure 3.

The identification and nomenclature of "potentially premalignant" lesions of the human breast have been a matter of controversy for many years. Among the non-invasive breast lesions, ductal carcinoma *in situ* (DCIS) is the most common and best characterized precursor to invasive carcinoma. Some researchers have also proposed models where there is a direct transition from normal to malignant epithelium, without any visible evidence of a preneoplastic stage (15). Nevertheless, evidence placing DCIS as a major precursor lesion of invasive carcinomas is substantial. For instance, the majority of IDCAs have an *in situ* component (16). Women with biopsy-proven DCIS have an increased risk for development of subsequent invasive breast cancer (17) and a high recurrence of invasive carcinoma occurs in women who have had breast-conserving treatment of DCIS (18). Taken together these findings strongly suggest *in situ* carcinomas are precursors to invasive carcinomas. A more insidious lesion is lobular carcinoma *in situ* (LCIS). LCIS is characterized by a uniform population of generally small and loosely cohesive cells growing in a solid occlusive fashion. Whereas the more common DCIS represents a highly heterogeneous group of lesions ranging from microscopic to grossly detectable intraductal carcinomas, as well as predominantly *in situ* carcinomas with areas of stromal microinvasion. When breast neoplastic cells begin proliferating outside their site of

origin, (i.e. beyond the containment of the basement membrane) and the microinvasion spreads further, the neoplasia is then termed invasive carcinoma. Invasive ductal carcinomas are not necessarily the result of DCIS nor are invasive lobular carcinomas the obligated direct result of LCIS. In fact, there is some evidence that LCIS may play an important role as a precursor of invasive ductal carcinoma as well (Figure 3) (19). It should be mentioned however that the relationship of LCIS as a precursor lesion of invasive breast cancer is a matter of controversy (19). It has been suggested that due to its morphologically diffuse and generalized nature, the presence of LCIS indicates that the whole breast epithelium is at risk of malignant transformation. It is possible that the distinction between DCIS or LCIS may be indicative of important differences in their genetic pathways during breast carcinogenesis.

Mammary epithelial hyperplastic lesions which demonstrate only some of the characteristics of *in situ* carcinoma are frequently identified in the clinic. These lesions are called atypical hyperplasias (AH) (20). Atypical hyperplasias can either be ductal (ADH) or lobular (ALH) in type. Finally, other less advanced hyperplastic lesions which are only associated with a slightly increased risk for breast cancer development, are known as proliferative disease without atypia (PDWA). PDWA lesions lack the qualitative and quantitative histologic features of AH (20) meaning that they comprise a variety of epithelial hyperplastic lesions from mild to florid but do not show signs of atypia.

## FAMILIAL AND SPORADIC BREAST CANCER FORMS

Family history constitutes the strongest known risk factor for development of breast cancer. Women who have a family tree in which several blood relatives were afflicted with breast cancer have a far greater chance of developing breast cancer as compared to the general population. Because of this fact, in the last few years intense research has focused on identifying the breast cancer susceptibility genes, passed down from generation to generation. However, the inherited forms of breast cancer account for only 5-10% of total breast cancer incidence (Figure 4). The remaining 90-95% of women who develop breast cancer do not appear to segregate for an inherited susceptibility allele. Such cases are known as "sporadic" breast cancer (21). This label is somewhat misleading since "sporadic" breast cancer is actually the most frequent form of breast cancer. Nevertheless, approximately 25% of breast cancer cases diagnosed before age 30 are believed to be caused by genetic factors alone (22). Analysis of familial pedigrees suggested the existence of various types of inherited breast cancers, consistent with models of autosomal dominant transmission of a highly penetrant susceptibility allele (21). The major familial breast cancer forms include (23):

- 1.) site-specific breast cancer, which is the most frequent and occurs in families in the absence of any other familial occurring neoplasm;
- 2.) breast and ovarian cancer syndrome, which is characterized by early-onset and high rate of bilaterality;
- 3.) Li-Fraumeni cancer syndrome, which is characterized by early-onset of breast cancer, bilaterality, and association with other

familial neoplasias, such as leukemia, sarcomas, brain cancer and adrenocortical carcinoma;

- 4.) Cowden disease which is a rare condition also known as multiple hamartoma syndrome and is characterized by multiple mucocutaneous hamartomatous lesions, both benign and malignant.

In addition to the above forms the Muir Torre Syndrome which is a variant of Lynch II syndrome, also includes breast cancer, although it is a very rare syndrome. This syndrome is caused by mutations in DNA mismatch repair genes and is associated with microsatellite instability (24,25).

## **BREAST CANCER SUSCEPTIBILITY GENES**

### ***BRCA1***

We have witnessed tremendous progress within recent years in the identification of genes responsible for several of the inherited breast cancer types. In 1990 genetic linkage analysis of affected families identified a gene predisposing individuals for early-onset breast cancer. This locus (i.e. Breast cancer 1 or *BRCA1*), was mapped to chromosome region 17q21 (26,27). Furthermore it was estimated that this tumor susceptibility allele, would account for 45% of families with high incidence of site-specific breast cancer and approximately 80% of families identified as carriers of the early-onset breast and ovarian cancer syndrome (28). After intense effort, the gene itself was cloned in

1994 by Miki and coworkers (29). Mutations in BRCA1 were found to cosegregate with the predisposing haplotype in affected kindred (29). Thus, a woman who has a mutation in the BRCA1 gene has a high risk of developing breast cancer and this risk increases over her lifetime reaching a peak by the age of 70 with a risk of 87% (28).

BRCA1 is a large protein with 22 exons and 1,863 amino acids and shows very little homology to other known genes and also has several alternative spliced forms. However, based on the fact that BRCA1 has a ring finger motif close to its amino-terminus and a leucine heptad repeat within its sequence, speculation was made that BRCA1 may function as a transcription factor (30). BRCA1 mutations are scattered throughout the entire coding region. Interestingly, a frequently found mutation (185delAG) is also found to be present in 1% of women from Ashkenazi Jewish descent (31). Most commonly the germinal mutations affecting BRCA1 are small insertions and deletions causing frameshifts, which produce stop codons, and result in truncation of the protein product.

The BRCA1 gene product appears to play a much smaller role, if any, in non-familial breast cancer. As with other tumor suppressor genes it was expected that mutations of BRCA1 would be frequent in sporadic breast cancer forms, particularly due to the common finding of 17q loss of heterozygosity in most breast tumors (32-35). However, no mutations in BRCA1 have been found in non-familial breast cancer cases. It has been suggested that subcellular mislocation of the BRCA protein may play a role in sporadic breast cancer. In normal breast epithelial cells, BRCA1 is localized in the nucleus, whereas in the majority of breast cancer cell lines and in malignant pleural effusions from breast cancer patients and in some primary tumors it is localized, mainly in the cytoplasm (36).

Some groups have suggested that BRCA1 is a secreted protein since it contains certain homology regions to granins, a protein found in secretory granules (37). Conflicting with this suggestion, the nuclear localization originally reported in normal cells was later confirmed by other groups (38). Some evidence has also accumulated indicating that normal BRCA1 may act as a tumor suppressor gene inhibiting tumor growth (26,39). At present the function of BRCA1 is controversial and therefore requires further analysis. However, both BRCA1 and BRCA2 have been shown to bind and colocalize in the nucleus with the DNA repair protein RAD51 but further analysis is needed to define BRCA's role in this pathway (38,40,41). Furthermore, it was recently suggested that BRCA1 is required for transcription coupled repair of oxidative damage (42). These investigators showed that cells deficient in BRCA1 are impaired in their ability to carry out transcription coupled repair of oxidative damage (42). This would imply that BRCA1 may be playing an important role as guardian of genomic integrity.

## *BRCA2*

A second breast cancer susceptibility gene was isolated in 1995, named BRCA2 (43). BRCA2 was originally mapped by linkage analysis to chromosome arm 13q12-13 (44). Similar to BRCA1, BRCA2 is a large gene encoding for 3,418 aminoacids and has several splice variants. Germinal mutations on this gene predispose a person to early-onset, site specific breast cancer and moderately predispose a woman to ovarian cancer. These families also present a higher incidence of male breast cancer and are associated with a higher predisposition to prostate, pancreatic, colon and

other cancers (43). As with BRCA1, the germinal mutations identified are spread throughout the coding sequence of BRCA2. Most of the mutations were frameshift mutations generating a truncated gene product as was the case with BRCA1. A particular BRCA2 germinal mutation, in this case 6174delT, is also frequently found (1%) in Ashkenazi Jewish women (45). Like BRCA1, BRCA2 appears to play no major role in sporadic breast cancer, since only very few somatic mutations were observed in these tumors. The function of the BRCA2 protein is uncertain as well.

Very recently the contribution of BRCA1 and BRCA2 to inherited breast cancer was assessed by linkage and mutation analysis in a series of 237 families with a history of breast cancer chosen at random without regard to the existence of other cancers (46). Linkage to BRCA1 was observed in 52% of the families, to BRCA2 in 32% and to neither in 16% of the families, indicating the existence of other predisposing genes. The vast majority (81%) of the breast/ovarian cancer families were associated with BRCA1 mutation, 14% due to BRCA2, while 76% of families with both female and male cancer cases were due to BRCA2. BRCA2 carriers appear to have a similar lifetime cancer risk as BRCA1 carriers but a lower risk before age 50 (46).

### *TP53*

Germline TP53 mutations have been found in affected families and shown to be causative of the Li-Fraumeni cancer predisposition syndrome (47,48). Breast cancer is one of the neoplasms affecting patients with this syndrome. In tumors from patients with Li-Fraumeni syndrome, loss of the wild-type TP53 allele is observed with retention of the mutant

allele. As indicated above, this syndrome is characterized by early-onset breast cancer, bilaterality, and association with other familial neoplasias, such as leukemia, soft tissue sarcomas, osteosarcoma, brain cancer and adrenocortical carcinoma.

Contrary to the previously described tumor susceptibility genes, TP53, a known tumor suppressor gene, has been shown to play an important role in sporadic breast cancer progression as well. However, germline mutations of this gene in the general population are rare. TP53, located on chromosome arm 17p13, is known to harbor somatic mutation in 25-45% of primary breast carcinomas (49). All evidence indicates that TP53 is one of the most frequently affected genes in breast cancer. The role of TP53 in breast carcinogenesis will be further discussed in a following section.

#### *PTEN / MMAC1*

Recently, a new putative tumor suppressor gene, PTEN, has been identified on chromosome 10q23.3. PTEN is responsible for Cowden disease's familial predisposition (50-52). Breast cancer is a component of this rare syndrome as was described in a preceding section. The majority of the women who have this mutated gene develop breast neoplasia and approximately half of these cases develop into breast cancer. The amino acid sequence of PTEN resembles two different types of proteins: tyrosine phosphatases, enzymes that remove phosphate groups from the amino acid tyrosine in other proteins; and tensin, a protein that helps connect the cell's internal skeleton of protein filaments to its external environment (53). Homozygous deletions and mutations affecting

PTEN have been found in both prostate and glioblastoma cancer cell lines. Somatic inactivating mutations of PTEN were associated with numerous primary prostate and endometrial carcinomas (54,55). However, practically no somatic mutations affecting PTEN were found in sporadic forms of breast cancer (56,57).

#### *ATM and HRAS (Putative breast cancer susceptibility genes)*

Several years ago it was suggested that heterozygous carriers of a defective Ataxia-telangiectasia (ATM) gene are at increased risk (3-5 fold) of developing breast cancer (58). However, later studies concluded that there was no clear association between the ATM mutants and the risk of early onset or familial breast cancer in general (59,60). Nevertheless, it is an issue of importance since ATM heterozygous carriers represent a high percentage of the population (0.5% - 5%). At this point, the role of ATM as a factor for increasing breast cancer risk in the general population is unclear.

Other potential breast cancer susceptibility alleles are the polymorphic variants of the HRAS gene minisatellite sequence (61,62). These studies reported a positive association between rare HRAS alleles and breast cancer (61,62). However, controversial results have been reported by other research groups (63) and further confirmation of the positive association between these rare alleles with breast cancer is required.

## *Common Enzymes Allelic Variants that may Contribute to Breast Cancer Risk*

Through molecular epidemiology studies, it is becoming apparent that the combination of a specific genetic makeup plus exposure to specific exogenous factors (e.g. environmental, chemical and physical carcinogens) play decisive roles in defining the risk for tumor development. The heterogeneity of the genetic background found in the general population would explain why certain individuals develop cancer while others do not even when exposed to a similar dose of a particular carcinogen (e.g. cigarette smoke and lung cancer risk).

Therefore, as previously suggested, breast cancer etiology may be explained by inherited predisposition to develop cancer, inherited predisposition to accumulate new mutations, and exogenous exposures (64) (Figure 1). This would be the basis for the expected etiologic heterogeneity found in the general population. Thus, although breast cancers are classified as a single disease, not all are caused by the same set of etiologic agents. Most likely, different population subgroups will respond differently to the same set of carcinogens.

Numerous studies have focused on a series of allelic variants that would confer increased tumor susceptibility. Several of these genes are enzymes involved in detoxification pathways that the organism utilizes to eliminate xenobiotics. In most cases, a phenotypic polymorphism in the metabolic rate of specific chemicals correlated with the finding of genotypic polymorphisms (i.e. allelic variants). The genotypic polymorphisms were detectable as restriction fragment length polymorphism (RFLP's) variants or as gene deletions. A good example of

such polymorphism affecting breast cancer is seen with the Cytochrome P-450 (CYP) superfamily of enzymes. These "Phase I" enzymes are responsible for the oxidative metabolism of diverse endogenous and exogenous substrates, such as steroids, prostaglandins, fatty acids, foreign chemicals and drugs. CYP enzymes are responsible for the biotransformation of xenobiotics to toxic intermediate metabolites (i.e. phase I). The level of CYP expression and CYP's catalytic activity can vary dramatically among the general population due to the highly polymorphic nature of these enzymes. The "Phase II" group of detoxification enzymes are also usually polymorphic and are responsible for the conjugation reaction necessary for the efficient excretion of toxic compounds. These enzymes transform toxic compounds into more hydrophilic forms for excretion. Ambrosone and Shields (65) have suggested that women who have genetic polymorphisms that could result in greater activation, or impaired detoxification of; aromatic and heterocyclic amines, (e. g. NAT1, NAT2, CYP1A2 enzymes); polycyclic aromatic hydrocarbons (e. g. GSTM1, CYP1A1 enzymes); and nitroso compounds (e. g. CYP2E1 enzyme) may be at greater risk for developing breast cancer. Recently it was reported that the GSTM1 homozygous null phenotype was associated with increased risk of developing breast cancer, similar associations were also observed with polymorphic variants of the enzymes GSTT1 and GSTP1 (66). On the other hand, it was very recently shown that cigarette smokers who are carriers of BRCA1 or BRCA2 mutations were found to have a lower breast cancer risk than subjects with mutations who never smoked indicating that somehow smoking appears to reduce breast cancer risk in these patients (67). This confuses the significance of published observations in the field and indicates the amount of work which lies ahead in order to clarify our

understanding of the interaction of environmental carcinogens and breast cancer genetic predisposition.

## **SOMATIC CHROMOSOMAL AND GENETIC ABNORMALITIES IN BREAST CANCER**

### **Cytogenetics of Breast Cancer**

Numerous studies have been performed in order to characterize the role of chromosomal abnormalities in breast cancer. However, as is the case with other solid tumors of epithelial origin, it has been difficult to identify any primary cytogenetic changes among the large number of apparently random alterations. This is due to the clonal heterogeneity characteristic of breast cancer as well as to the inherent difficulties in obtaining high-quality metaphases from solid tumors. Nevertheless, the prevalence of several specific chromosomal aberrations have been noted. The most frequent tend to be numerical alterations of whole chromosome copy number including trisomies of chromosomes 7 and 18 and monosomies of 6, 8, 11, 13, 16, 17, 22, and X (68). The most common aberrations in non-metastatic near-diploid tumors are, loss of chromosomes 17 and 19, trisomy of chromosome 7, and overrepresentation of chromosome arms 1q, 3q, and 6p (69). Structural alterations include terminal deletions and unbalanced nonreciprocal translocations, most frequently involving chromosomes 1, 6, and 16q. Breakpoints for structural abnormalities cluster to several chromosomal segments, including 1p22-q11, 3p11, 6p11-13, 7p11-q11, 8p11-q11, 16q, and 19q13 (69). In particular, 16q was shown to participate systematically in

translocations with chromosome 1q and to display frequent deletions. In fact, some investigators have suggested that specific abnormalities affecting chromosome 16q could be considered primary cytogenetic aberrations since they were observed in the absence of other anomalies (70,71).

A recently developed molecular cytogenetic technique called comparative genomic hybridization (CGH), allows for the analysis of chromosome copy number abnormalities involving segments of at least 10 Mb (72). Since CGH involves hybridizing differentially labeled genomic DNA from a tumor and a normal cell population to the same normal metaphase, it circumvents some of the difficulties encountered in conventional karyotyping. Through such analyses, nearly every tumor analyzed revealed increased or decreased DNA sequence copy number (73).

The most common regions of increased copy number in breast cancer as determined by CGH include 1q, 8q, 17q22-24, and 20q13. Regions of decreased DNA copy number were also observed and include 3p, 6q, 8p, 11p, 12q, 13q, 16q, and 17p (74). For some of these regional losses, candidate genes exist that may be the target of deletion in the progression to a malignant phenotype (Table 1). These genes and their corresponding regions will be discussed in more detail in the following sections. Interestingly, when both loss and gain of DNA copy number as determined by CGH were compared with survival data in a series of node negative breast tumors, only copy number losses were significant for recurrence and for overall survival (75). However, as is the case with conventional cytogenetics, CGH studies failed to reveal any characteristic abnormalities that occur in the majority of breast tumors or to identify any abnormalities which could be considered "primary".

## **Oncogenes and Gene Amplification**

In human breast cancer as in other solid tumors, the most common aberration affecting oncogenes appears to be gene amplification. Abundant evidence demonstrates that huge regions of DNA, up to entire chromosome arms, can be amplified as a contiguous unit. The importance of this to breast cancer development is still unclear although it suggests that genes within these regions are overexpressed, due to their high representation. Chromosomal regions overrepresented in tumor cells suggest the presence of activated oncogenes. Proto-oncogenes encode proteins involved in cascade of events leading to growth in response to mitogenic factors. Alteration in the normal function of proto-oncogenes, through mutation or increased expression, can result in a constant growth stimulus and a constitutive mitogenic response. Aberration of a single allele of an oncogene can be sufficient to lead to altered signal and as such is dominant. Current data suggest that of the numerous oncogenes described to date, only a few may have a role in breast tumorigenesis.

## **Regions Affected by Gene Amplification**

### *Region 17q12 (ERBB2)*

In 1987, ERBB2 was demonstrated to be overexpressed and amplified in 20-40% percentage of breast cancers (76,77). Amplification was shown to be consistently accompanied by increases in mRNA and protein levels (76,77). In later studies, increased copy number of the long arm of

chromosome 17 (17q) which contains the ERBB2 gene, demonstrated a 50 to 100 fold amplification in some cases as determined by gene fluorescence *in situ* hybridization analysis (72).

The fact that ERBB2 is overexpressed in a high percentage of breast cancers implicates its involvement in breast tumorigenesis (78,79). Therefore in recent years the diagnostic and possible treatment value of ERBB2 detection has been extensively studied. Early studies reported a prognostic value of ERBB2 overexpression in node negative breast cancer. However, more recent studies using larger data sets did not support these early observations and question the prognostic role for ERBB2 expression in node positive breast cancer. Expression of ERBB2 may have value in predicting response to specific therapies, additional studies are underway to confirm these observations (reviewed by Ravdin & Chamness 1995). For instance, ERBB2 overexpression has been associated with increased resistance to chemotherapy (82) and estrogen receptor positive patients who overexpress ERBB2 are less likely to respond to hormone therapy (83).

Studies have also shown that activation or overexpression of ERBB2 in transgenic mice results in the genesis of mammary tumors (84). Whereas neutralizing antibodies against ERBB2 lead to tumor regression (85). The possibility of using antibodies against ERBB2 as a means of treating breast cancer is presently under investigation (86,87).

The ERBB2 proto-oncogene is a member of the epidermal growth factor receptor family. All of the family members which include EGFR, ERBB2 (Her-2/neu) ERBB3 and ERBB4, have demonstrated overexpression in breast cancer. In addition, overexpression of ligands for these receptors, such as TGF-alpha, have been associated with neoplastic transformation in transgenic mouse models (88). This family of receptors encodes

transmembrane glycoproteins with tyrosine activity. However, although the family members share high homology their ligand specificity is distinct. It has been suggested distinct biochemical and biological responses of the individual receptors such as ERBB3 and ERBB4 (89). Therefore although these receptors may show similarity in their ability to regulate cell proliferation their mechanism of action is most likely diverse.

#### *Region 8q (c-Myc)*

Amplifications at region 8q including the oncogene *c-myc*, a gene known to be overexpressed, either by amplification or regulatory means, in breast cancers (90). *c-myc* is a member of a small family of related proteins that function as sequence specific transcription factors (91). Activation of the *c-myc*, *Nmyc* and *Lmyc* genes has been described in many human cancers (92). In normal cells, *c-myc* expression is rapidly induced following mitogenic stimulation, and its activity is absolutely dependent upon the presence of growth factors (93). The *c-myc* protein is commonly implicated in mediating the transition of cells from quiescence to proliferation (94). Therefore, *c-myc* is considered to be a positive regulator of cell growth and its activation is thought to confer a growth advantage upon a tumor cell. Conversely, *c-myc* has also been demonstrated to induce apoptosis a function more consistent with a negative regulator of cell growth (95,96). A possible explanation for this bifunctional activity lies in the fact that in order for *c-myc* to act as a promoter of cell proliferation appropriate serum growth factors, stimulating growth via a separate pathway, must be present (92). In the

absence of growth regulators, over expression of *c-myc* is sending conflicting signals to the nucleus, which in turn initiates programmed cell death (i.e. apoptosis).

The *myc* gene has been shown to be amplified in approximately 25% of breast carcinomas (90). Overexpression of *c-myc* in transgenic mice results in mammary tumors (97), and amplification of *c-myc* has been associated with high grade tumors in humans (98). Of additional interest, *c-myc* expression is modulated by the presence of estrogen in estrogen-responsive cell lines, and constitutively high *c-myc* expression is observed in hormone-dependent lines, probably because of increased stability of the transcript (99).

#### *Region 11q13 (cyclin D)*

Chromosome region 11q13 has also been reported to be amplified in 15-20% of breast cancers and is associated with poor prognosis (100). The *cyclin D1* gene, located in this region, is thought to be the target of such amplification. Cyclin D1 is overexpressed in 45% of breast carcinomas, most of which are both estrogen and progesterone receptor positive (101,102). Studies show that transgenic mice homozygous null for *cyclin D1* fail to undergo proliferative changes of the mammary epithelium associated with pregnancy, thereby indicating a role for *cyclin D1* in steroid-induced proliferation of the mammary epithelium (103). Transgenic mice overexpressed *cyclin D1* have been shown to develop mammary carcinomas (104). Analysis of *cyclin D1* expression by mRNA *in situ* hybridization has shown a dramatic increase of *cyclin D1* expression

in 76% of low grade carcinoma *in situ*, further suggesting a role for cyclin D1 in the tumorigenesis of the breast (105).

Mapped within this same region is the *int-2* gene. Transgenic mice strains containing the *int-2* transgene develop multifocal preneoplastic hyperplasia of the mammary gland which can give rise to focal mammary tumors (106). The possibility that an additional gene responsible for breast tumorigenesis within this amplified region is *int-2* is controversial since corresponding increases in mRNA and protein levels for *int-2* rarely correspond to amplification status. Thus, the possibility remains that a yet unknown gene located close to *int-2* might have a biological effect, whether *int-2* itself is merely co-amplified remains to be seen.

#### *Region 20q13 (AIB1)*

The gene (AIB1), amplified in breast cancer, was very recently identified as a leading candidate for the amplification of region 20q13 (region described to be amplified in 15-30% of cases) (107). AIB1 was found amplified in all estrogen receptor positive cell lines and it has been identified as a nuclear steroid receptor coactivator (107). AIB1 amplification may contribute to the development of steroid-dependent breast cancers by interacting with the estrogen receptor to enhance the effects of estrogen on tumor cells.

## **Tumor Suppressors and Loss of Heterozygosity**

Knudson, on the basis of statistical analysis of clinical observations, was the first to suggest that retinoblastoma was a cancer caused by two mutational events (108). In the hereditary form of retinoblastoma one mutation is germinal; thus only a single additional somatic mutation is required. This resulted in early onset and a tendency toward bilateral tumorigenesis. In the sporadic form, both mutations are somatic, resulting in a tendency toward unilaterally and late onset. It was later suggested that these two mutational events could occur within separate alleles of a regulatory gene (109). Supporting this, cytogenetic analysis of retinoblastoma revealed characteristic deletions of the long arm of chromosome 13. Subsequent analysis of the same chromosome region led to the cloning of *RB1* and identification of aberrant transcripts encoded from the remaining allele (110). As a consequence of these studies, a precedent emerged where inactivation of one allele of a tumor suppressor is accomplished by mutation, leading to the eventual deletion of the remaining normal allele through chromosomal aberrations and thus loss of heterozygosity (LOH) is thereby observed in the suppressor locus. This precedent is now considered the convention for tumor suppressor gene inactivation and similar observations have been made for several other putative tumor suppressor genes (e.g., *APC*, *DCC*, *VHL*, *TP53*; reviewed by (111). Therefore, LOH is considered indirect evidence for the existence of a suppressor gene within the affected chromosomal region.

## *Breast Cancer Allelotype*

The chromosomal mechanisms by which loss of heterozygosity occurs tend to involve large segments of DNA, thus it is possible to utilize adjacent genes or known noncoding sequences as markers to identify deleted regions harboring putative suppressor genes whose loss may be important in the genesis or progression of a tumor. One such genetic marker is the naturally occurring simple sequence length polymorphisms (SSLPs). SSLPs consist mainly of dinucleotide repeats, primarily (CA)<sub>n</sub>, which are repeated in tandem at variable number interspersed throughout the genome (112). These polymorphic microsatellites have a mean heterozygosity of 70% and recent mapping efforts reported an average spacing of 199 Kb (113). Through known linkage maps and comparison to physical maps, it is possible to select highly polymorphic microsatellites at any position within the genome. Further, through PCR amplification of these microsatellites and comparison with normal DNA from the same patient, it is possible to generate a comprehensive map of allelic imbalances and losses (allelotype) occurring in a neoplasm.

Numerous studies have analyzed the breast cancer allelotype, and numerous regions of allelic imbalance have been described using microsatellites as well as the older restriction fragment length polymorphism analysis. Deville and Cornelisse, reviewed data from more than 30 studies revealing a consensus of imbalances affecting 12 chromosome arms at a frequency of more than 25% (Table 1). Chromosome arms 1p, 1q, 3p, 6q, 8p, 11p, 13q, 17q, 18q, and 22q were affected at a frequency of 25-40%, whereas chromosome arms 16q and 17p were affected in more than 50% of tumors (68). In addition, chromosome arm

9p, has recently been reported to be affected by allelic imbalances and losses in numerous breast carcinomas (114). In general, the loss of genetic material in many of these regions has been corroborated by either CGH or classic cytogenetic data (68,115). Some of these regions are known to harbor tumor suppressive genes whose loss has been demonstrated through a variety of techniques, including Southern blot analysis and FISH using gene-specific single-copy probes (111).

Although there is overwhelming evidence that these genetic losses occur, inherent difficulties exist in determining the relevance of such losses to breast carcinogenesis. In most cases, the tumors analyzed were of the invasive type and/or advanced stages of progression, leading to the question of whether these losses are causative factors of tumorigenesis or consequences of the general genomic instability inherent to tumors. It is possible that certain losses may be selected for in the progression or clonal evolution of a tumor to a more advanced type but not strictly necessary for the genesis of the tumor. Some of these questions could be addressed in part through comparative allelotyping of both noninvasive and invasive tumors.

The relative timing and frequency of allelic losses of commonly affected regions in breast cancer was estimated by comparing the allelotype of preinvasive ductal carcinomas (DCIS) and invasive carcinomas (81). The allelotypic analysis of DCIS samples revealed that chromosomal regions 3p, 3q, 6p, 11p, 16p, 18p, 18q, and 22q were not affected by a high frequency of loss, on the other hand analyses of these same regions of invasive tumors showed them to be affected in 10-40% of cases (81) (Table 1). These findings are in agreement with those of Radford et. al. who examined 61 DCIS samples (116). Since allelic losses

affecting these regions were not frequently observed at the noninvasive (DCIS) stage it can be concluded that alterations of these regions are late events in breast cancer progression. More importantly, allelic imbalances observed on chromosome arms 7p, 7q, 16q, 17p, and 17q (81), as well as 9p as reported by others (117), appear to be early abnormalities because they occur frequently in DCIS (Table 1).

### **Targets of Allelic Loss**

#### *Chromosome Region 17p13*

The short arm of chromosome 17, is subject to allelic loss in more than 50% of invasive ductal carcinomas, and approximately 30% of noninvasive ductal carcinomas (81,116,118). This high frequency of allelic loss suggested that a tumor suppressor of relevance to breast tumorigenesis resides in this region. Indeed, tumor suppressor *p53* maps to chromosome band 17p13 and is known to harbor somatic mutation in 25-45% of primary breast carcinomas (49). Recently *p53* mutations were identified in mammary ductal carcinoma *in situ* but not in epithelial hyperplasia (119). It has been suggested that *p53* mutation analysis may serve as a marker for identifying preinvasive lesions at increased risk of developing invasive carcinoma.

We have already discussed the relevance of germinal *p53* mutations as the cancer predisposing alteration in the Li-Fraumeni syndrome (47,48). In tumors from patients with Li-Fraumeni syndrome, loss of the wild-type allele is observed in conjunction with retention of the mutant *p53* allele. Functional studies of cells with mutant *p53* indicate a change

of phenotypes, including cellular immortalization, loss of growth suppression, and fourfold increase in protein half-life which leads to *p53* accumulation. Accumulation of *p53* protein, observed by immunohistochemical analysis in roughly 30-50% of sporadic breast carcinomas, was proposed to be an indicator of higher risk of recurrence in patients with tumors positive for *p53* expression (reviewed by (120)). It appears that *p53* inactivation through mutation and LOH is intrinsically linked to the development of subsequent further genomic instability as suggested by *in vitro* findings, as discussed in a separate chapter, and as demonstrated in experimental models of mammary cancer (121).

Although *p53* is most likely the driving force for allelic loss on 17p, some reports indicate that there may exist another distinct locus that may be a target of allelic loss. In an analysis of 141 breast tumors, Cornelis et al. observed a strong association between *p53* mutation and allelic loss of the *p53* locus (122). However, in cases where *p53* mutation was not observed, allelic loss of distal region 17p13.3 was always observed, sometimes without *p53* allele loss. Similar findings of distal deletion of 17p were also observed in DCIS (116). While these findings support the existence of a second gene as target of allelic loss, further studies are needed to address this issue.

#### *Chromosome Region 17q21-22*

The long arm of chromosome 17, also frequently affected by allelic imbalance in both familial and sporadic breast cancers, has recently been subjected to extensive analysis because 17q has been linked to familial breast cancer (123). As a result, the *BRCA1* gene was isolated by

positional cloning as discussed in preceding sections (29). However, when sporadic breast tumors with allelic loss of 17q were examined for *BRCA1* coding sequence alterations, only about 10% of those with LOH revealed any change of sequence, and those mutations were found to be germinal (124).

Another known putative suppressor gene localized in this region, nm23 or *NME1*, has been shown to undergo allelic loss in as much as 60% of breast carcinomas (125). However, analysis of *NME1* has not revealed evidence of mutations (126). An additional possible explanation for allele loss in 17q is the existence of a yet-unidentified gene within this region as the target for LOH (122).

#### *Chromosome Region 13q14*

Loss of the *RB1* region 13q14 has been reported for numerous neoplasms including small cell lung carcinoma, bladder carcinoma, osteosarcoma, and breast carcinoma (reviewed by (111). These losses appear to be relatively early losses in some tumors since 15-20% of tumors at the DCIS stage reveal allelic loss of 13q (81,116). However, when allelic loss and expression are examined in the same breast tumors, no correlation between the two is observed, suggesting that Rb inactivation is not acquired by allelic loss and that another gene may be the target of such inactivation (127). As discussed in a previous section, a second breast cancer susceptibility gene, *BRCA2*, was mapped to chromosome 13q12-13 (43). This suggested that the *BRCA2* gene may be involved in sporadic breast cancer as well. However, similar to the findings with *BRCA1* on 17q, when sporadic breast tumors were analyzed

for mutation of *BRCA2*, mutations were infrequent, indicating that *BRCA2* is not the gene being targeted by loss (128-130). Brush-1 is another gene that has been mapped to 13q12-13, proximal to *RB1*. Analysis of Brush-1 expression showed it to be low to absent in 6 of 13 breast cancer lines and decreased in four of four tumors showing LOH of 13q12-13 (131). However, no sequence analysis has yet been reported, and the question of whether decreased expression of Brush-1 results from allelic loss involving large regions of another gene has yet to be addressed.

#### *Chromosome Region 16q*

Chromosome 16q has been suggested as a site for the occurrence of primary cytogenetic structural abnormalities in the development of breast cancer (70,71). In particular the long arm of chromosome 16 was shown to systematically participate in nonrandom translocations with chromosome 1. Breast cancer allelotypic studies have also shown the common occurrence of allelic losses affecting the long arm of chromosome 16 (132-134). Several studies have reported the occurrence of frequent allelic losses affecting chromosome 16q in DCIS (81,116,133).

It has been suggested that more than one putative tumor suppressor resides in the chromosome region 16q. At least two regions of chromosome 16q have consistently been reported to show LOH: 16q21 and 16q24.2-qter (132-134). In most recent studies, high-resolution allelotyping of chromosome 16 in DCIS lesions have identified three distinct regions with a very high incidence (about 70% or more) of allelic losses (41). Two of the three regions agree with previously described areas: 16q21 at locus D16S400 and 16q24.2 at locus D16S402 (41).

However, the region with the highest incidence of LOH observed, lies within 16q23.3-q24.1 close to marker D16S518 (41).

E-cadherin (CDH1), a cell adhesion molecule implicated as an invasion suppressor protein, is one possible candidate target of the LOH at chromosome 16q21. Interestingly, this gene was demonstrated to be mutated at a high frequency in invasive lobular carcinomas of the breast. The lack of E-cadherin expression is believed to be the cause behind the infiltrative growth pattern characteristic of lobular carcinomas (135). However, the more common, invasive ductal carcinomas do not show high incidence of E-cadherin mutations. In addition to mutation E-cadherin may be inactivated by CpG methylation within the gene's promoter region (136,137). Expression of a second cadherin gene, H-cadherin, map to region 16q24 (138) was reported to be absent or reduced in several breast cancer cell lines. Further studies are necessary to identify additional possible targets for the common allelic losses observed to affect this autosome in breast cancer.

#### *Chromosomal Region 9p*

Chromosomal region 9p21, as previously discussed, has been shown to be affected by allelic loss or imbalance in more than 58% of invasive ductal carcinomas and 30% of DCIS, suggesting it may be involved in breast tumorigenesis (114,117). Previously, the p16 tumor suppressor gene was identified within this region by positional cloning and shown to be affected by homozygous deletions in 60% of breast carcinoma lines (139). However, when primary breast tumors were analyzed for mutation of the *CDKN2* coding region, few mutations were found (114). More recent

analysis, including FISH determination of gene copy number, methylation of the 5' region, and analysis of expression, indicate that *p16* appears to be a target of abnormalities in approximately 40% of breast tumors (140). These observations substantiate a role for *p16* inactivation in the tumorigenesis of the breast and as a target of 9p allelic loss. Interestingly, however some breast tumors show overexpression of *p16* indicating that involvement of this gene as well as that of *p14ARF* (homolog of mouse *p19ARF*), encoded at the same locus in an alternative reading frame is more complex than previously thought, as will be discussed in the following section.

## **CELL CYCLE IN BREAST CANCER**

Cell replication in eukaryotes proceeds through an orderly cascade of events manifested as the cell cycle. The machinery responsible for such progress includes a hierarchy of proteins and complexes each exerting an effect on the next. At the top of this hierarchy are the cyclin subunits, whose expression and stability oscillate in a phase-dependent manner. The expression of certain cyclin genes can be upregulated by different mitogenic stimuli, for example, the upregulation of cyclin D1 by estrogen (141). Each of these cyclins can associate in a specific manner with corresponding cyclin-dependent kinases (CDKs). Cyclins are in competition with CDK inhibitors, which have the ability to displace the cyclin and form an inactive complex with the CDKs. When CDKs are active, they phosphorylate, and hence inactivate, other proteins with transcription-repressing activity (Reviewed by Sherr, 1996) (Figure 5).

Of the restriction points, G1 to S is best characterized in breast cancer. Key players in early G1 and after the passage of cells from G0 to G1, include cyclins D1-D3, CDKs 4 and 6, the specific inhibitors of these CDKs, p15, p16, p18 and p19 and the substrates of CDKs, Rb and Rb-like proteins. Later in G1 and fueled by E2F1 transcriptional activation, cyclin E and its partner CDK2, become important players in the G1-S transition. Collectively, these proteins are known elements responsible for regulating progression through G1 and as a consequence loss of function or abnormalities in the expression of an individual protein can lead to cell cycle dysregulation and altered cell proliferation.

An additional family of CDK inhibitory proteins also exists which includes, p21<sup>cip1</sup>, p27<sup>kip1</sup> and p57<sup>kip2</sup>. Of the proteins mentioned, the Rb protein, cyclin D1, cyclin E, p16 and p27 have all been observed to be affected in breast carcinogenesis. As previously mentioned, cyclin D1 has been shown to be both amplified in 10-20% of breast tumors and overexpressed in the majority of breast tumors (101,102,105). Cyclin D1 competes with *p16* for heterodimerization with the CDKs. When cyclin D1 is more abundant than p16, it binds to and activates CDK4 and CDK6 (Figure 5). Recently cyclin D1 mRNA and estrogen receptor expression were found to be positively correlated in primary breast cancer (144). There is no conclusive evidence however demonstrating that estrogen receptor directly up regulates cyclin D1 transcription.

Recent studies have suggested that overexpression of cyclin E, which is often found in breast tumors, is functionally redundant to cyclin D (145). In cells overexpressing both cyclin E and p16, cyclin E can functionally replace cyclin D providing tumor cells with a growth advantage (145). They do this by activating CDK's which in turn

phosphorylate *Rb*, releasing E2F and initiating gene transcription, leading to cell cycle progression and a self-perpetuating positive regulatory loop (Figure 5). Interestingly, it was recently reported a bad prognosis and very high mortality rate in women with breast cancers showing high cyclin E expression concomitant with low expression of the CDK inhibitor p27<sup>kip1</sup> (146).

Inactivation of *Rb* itself has been described in breast cancer as a means of enhancing cell cycle progression. Although, when multiple modes of inactivation are accounted for, *Rb* is inactivated in less than 20% of breast cancers (127,147). In the vast majority of tumor lines there is an inverse relationship between *Rb* and *p16* expression (148,149). (i.e., breast tumor cell lines which retain *Rb* expression have no expression of *p16*. Whereas, cell lines retaining *p16* expression often lack expression of *Rb*.) When primary breast tumors were analyzed for *p16* expression, approximately 50% showed loss or reduced expression (140). This loss of expression may be due to homozygous deletion, methylation or in rare instances mutations (140).

The CDK inhibitor *p21 CIP1* as indicated, is a universal inhibitor of CDKs, inducing cell cycle arrest at both the G1/S and G2/M restriction points (150). Inhibition of DNA replication occurs when *p21* complexes with the proliferating cell nuclear antigen, PCNA (151). Because *p21* gene transcription is regulated by *p53*, it has been suggested that *p53*-dependent cell cycle arrest is mediated by *p21*. Indeed, *p21* nullizygous mice fibroblasts fail to undergo G1 arrest when *p53* is activated following DNA damage, although apoptosis is unaffected and occurs when *p53* is activated in these same cells (152).

As previously mentioned, positive detection of the p53 protein accumulation has been shown to be associated with p53 mutations and a higher risk of breast cancer recurrence (reviewed by Ozbun and Butel, 1995). Therefore, p53 inactivation appears to be a critical event in the tumorigenesis of the breast. This also suggests that an additional consequence of p53 inactivation would be abrogation of cell cycle arrest through loss of transcriptional activation of p21.

Interestingly, very recently it was demonstrated an important regulatory link between both the P53 and Rb pathways. At the center of this link is the recently described p14ARF (previously called p19ARF because of the mouse homolog). As mentioned in a preceding section, this gene is encoded at the INK4a locus in chromosome 9p21, as an alternative reading frame of the cyclin dependent kinase inhibitor p16<sup>INK4a</sup>. It was demonstrated that the putative tumor suppressor ARF gene physically interacts with MDM2 and as a consequence basically blocks MDM2-induced p53 degradation and transcriptional inactivation (153,154). Thus, this interaction leads to increase p53 stability and accumulation (Figure 5). Further strengthening the connection between the p53 and Rb pathways, it was very recently reported that ARF is transcriptionally upregulated by E2F1 (155). This allowed to speculate that perhaps abnormal cell proliferation, which results in E2F1 increase (Figure 5), in turn would result in increase of ARF which would lead to cell arrest or apoptosis via p53. This would not happen if an additional abnormality takes place such as p53 mutation or ARF inactivation (155). In studies from our laboratory we have demonstrated that both ARF and p16 expression levels are highly variable in breast cancer (140). We observed subsets of tumors that lack expression of both genes (p16 and ARF) due to common inactivation events

such as homozygous deletion. On the other hand we have also observed numerous tumors that dramatically overexpressed ARF. We are currently addressing the p53 status in the same tumors.

## **APOPTOSIS AND BREAST CANCER**

Closely linked to the deregulation of the cell cycle are the molecular pathways leading to programmed cell death (i.e. apoptosis). When DNA is altered during replication, control checkpoints stop the mitotic cycle in order to repair the damage. If the DNA cannot be repaired, apoptosis is induced. At first much of cancer research focused on uncontrolled cell proliferation, now researchers are aware of the important role cell death has in maintaining homeostasis.

Much remains to be done in terms of understanding the sequence of events that occur during apoptosis, which of them are essential of the process and their role in carcinogenesis. As already mentioned, p53 plays an important role in directing cells into apoptosis when DNA damage occurs. Other key players include the family of Bcl proteins and ICE (interleukin 1 $\beta$  converting enzyme).

Bcl2 is a potent repressor of apoptosis conferring survival advantage to cells expressing it. On the other hand, Bax, another Bcl2 family member, is capable of countering the death repressor activity of Bcl2. Bax has been found to exist as a homodimer and is also capable of forming heterodimers with Bcl2 *in vivo* (156). Therefore the ratio of Bax to Bcl2 in a given cell may dictate whether the cell survives upon receiving apoptotic stimuli. This type of homeostatic control by the ratio of homo and heterodimers can be expanded to include several members of

the Bcl2 family. Some Bcl2 members acts as promoters of apoptosis (i.e. Bax) while other members inhibit apoptosis (i.e. Bcl2). Although not a member of the Bc12 family, cMyc has been shown to cooperate with Bcl2 to achieve immortalization of tumor cells (92). Bcl2 protein expression is highly variable in breast cancer and it was suggested that high expression associated with favorable clinicopathological features (157,158). Recently a study analyzed Bcl2, Bax, Bcl-x and Bak expression and loss of apoptosis in small, non-metastatic breast carcinomas (159). Bcl-2 expression but not Bclx expression was associated with loss of apoptosis. Expression of Bax and Bak was found significantly associated with increased apoptosis in the breast carcinomas. These large gene families form complex set of interactions which may balance the scale either towards or away from apoptosis. It is still premature however to determine whether alterations in pathways of apoptosis play a relevant role in breast carcinogenesis.

## **ESTROGEN AND BREAST CANCER**

The role of estrogen as an important factor in the etiology and progression of human breast cancer has been well documented. It was already observed 100 years ago that ovariectomy could lead to breast cancer regression in premenopausal patients (160). The extent of exposure to ovulatory cycles is one of the most important endogenous causes associated with a higher risk for development of sporadic breast cancer (4). However, while the association of estrogen in the development of breast cancer is well established, the fundamental mechanism(s) by

which this hormone modulates cell growth and tumor development are not yet clear.

It is known from *in vitro* and *in vivo* studies that estrogen's mechanism of action is via its ability to bind the estrogen receptor (ER) which in turn binds specific enhancer regions on the DNA and regulates gene transcription (161). The interaction of estrogen with its receptor and the recruitment of accessory cofactor proteins to bind DNA and activate gene transcription has been the focus of intense recent research (162). However we understand very little downstream from these events. Important questions still remain such as; What are the main gene targets upon which estrogen acts to exert a growth response? What is the chronology of such events?

Estrogen has been shown to increase the pool of cells synthesizing DNA by recruiting non-cycling cells into the cell cycle and by reducing the length of the G1 phase (163). The ability of estrogen to regulate the transcription of c-Myc and c-Fos is believed to be, in part, responsible for estrogen's stimulatory effects on the cell cycle (164). Entry into S phase was found to be preceded by increased activity of both Cdk4 and cyclin E-Cdk2 and hyperphosphorylation of pRB, all within the first 3-6 hours of estradiol treatment (165). The increase in Cdk4 activity was accompanied by increases in cyclin D1 mRNA and protein, indicating that an initiating event in the activation of Cdk4 was increased cyclin D1 gene expression. In addition to cyclin D1's ability to activate cdk4, cyclin D1 has recently been shown to directly enhance transcription of estrogen receptor related genes (166). Cyclin D1 does this without binding cdk4 and in the absence of estrogen, thereby identifying an additional role for cyclin D1 in promoting cell growth.

However the effects of estrogen on cyclin D1 expression may not be a direct effect of the estrogen receptor on the cyclin D1 gene promoter. The ability of protein synthesis inhibitors to abolish cyclin D mRNA induction by estrogen, suggests intermediary proteins could be involved (165). Clearly our understanding of how estrogen exerts its effects on breast tissue requires further analysis.

## **NEW TECHNOLOGIES AND FUTURE DIRECTIONS**

It is estimated that within the next few years we will have compiled gene sequence information for the entire human genome. However if we are to use the tremendous amount of information gained to improve the treatment of breast cancer patients, it is imperative that we bridge the gap between genes and their relationship to a particular physiopathological outcome. To date the majority of molecular biology research has focused on abnormalities of the genome such as mutation, gene amplification and loss of heterozygosity as discussed in preceding sections. Identifying defects in the genome associated with breast cancer is the first level of genomic complexity. The next level of complexity is characterizing the changes in gene expression as a cell progresses from normal to malignant.

Present day advances in gene expression technology are allowing researchers to study this next level of genomic complexity by defining global changes in gene expression. Technologies such as SAGE (Serial Analysis of Gene Expression) and Microarray technologies are at the cutting edge of cancer research. Ultimately, the ability to understand the detailed mechanisms of tumor progression, from the very early stages of

carcinogenesis through metastasis will allow researchers to identify key components and interactions of the malignant pathway. Recently, the feasibility of SAGE was demonstrated by analyzing more than 300,000 transcripts derived from at least 45,000 different genes in both normal and neoplastic cells (167).

The knowledge found by defining global and specific alterations in the transcription of premalignant and malignant cells, would allow researchers to concentrate on gene targets that will better serve as diagnostic and prognostic tools. Ultimately it would be ideal to achieve a very precise matching of treatment to individual tumors profiles. A logical additional consequence will be the designing of more rationale therapeutic approaches.

## REFERENCES

1. Feuer, E., Wun L., Boring C., Flanders W., Timmel M. and Tong T. (1993) The lifetime risk of developing breast cancer. *J Natl Cancer Inst*, **85**, 892-897.
2. Kelsey, J. and Horn-Ross P. (1993) Breast Cancer: Magnitude of the problem and descriptive epidemiology. *Epidemiol Rev*, **15**, 7-16.
3. Wingo, P.A., Tong R. and Bolden S. (1995) Cancer Statistics, 1995. *CA-A Cancer Journal for Clinicians*, **45**, 8-30.
4. Pike, M., Spicer D., Dahmoush L. and Press M. (1993) Estrogens, progesterones, normal breast cell proleferation, and breast cancer risk. *Epidemiol Rev*, **15**, 17.

5. McGregor, H., Land C., Choi K., Tokuoka S., Liu P., Wakabayashi T. and Beebe C. (1977) Breast cancer incidence among atomic bomb survivors, Hiroshima and Nagasaki, 1960-1969. *J Natl Cancer Inst*, **59**, 799.
6. Bhatia, S., Robison L., Oberlin O., Greenberg M., Bunin G., Fossati-Bellani F. and Meadows A. (1996) Breast cancer and other second neoplasms after childhood Hodgkin's disease. *N Engl J Med*, **334**, 745-751.
7. el-Bayoumy, K. (1992) Environmental carcinogens that may be involved in human breast cancer etiology. *Chem Res Toxicol*, **5**, 585-590.
8. Wolff, M. and Weston A. (1997) Breast cancer risk and environmental exposures. *Env Health Perspect*, **4**, 891-896.
9. Russo, J., Rivera R. and Russo I. (1992) Influence of age and parity on the development of the human breast. *Breast Cancer Res Treat*, **23**, 211-218.
10. Wellings, S., Jensen H. and Marcum R. (1975) An atlas of subgross pathology of 16 human breasts with special reference to possible precancerous lesions. *J Natl Cancer Inst*, **55**, 231-275.
11. Rosen, P.P. (1979) The pathological classification of human mammary carcinoma: Past, present and future. *Ann Clin Lab Sci*, **9**, 144-156.
12. Tavassoli, F. (1992) *Pathology of the Breast*. Appleton & Lange, Norwalk, CT.
13. Silverstein, M., Lewinsky B., Waisman J., Gierson E., Colburn W., Senofsky G. and Gamagami P. (1994) Infiltrating lobular carcinoma.

It is different from infiltrating duct carcinoma? *Cancer*, **73**, 1673-1677.

14. Dixon, J.M., Anderson T.J., Page D.L., Lee D., Duffy S.W. and Stewart H.J. (1983) Infiltrating lobular carcinoma of the breast: an evaluation of the incidence and consequence of bilateral disease. *Br J Surg*, **70**, 513-516.
15. Ponten, J., Holmberg L., Trichopoulos D., Kallioniemi O., Kvale G., Wallgren A. and Taylor-Papadimitriou J. (1990) Biology and natural history of breast cancer. *Interntl J Cancer*, **5**, 5-21.
16. Alpers, C. and Wellings S. (1985) The prevalence of carcinoma in situ in normal and cancer-associated breasts. *Human Pathology*, **16**, 796-807.
17. Dupont, W. and Page D. (1985) Risk factors for breast cancer in women with proliferative breast disease. *N Engl J Med*, **312**, 146-151.
18. Solin, L., Recht A., A F., Kurtz J., Kuske R., McNeese M., McCormick B., Cross M., Schultz D., Bornstein B. and al e. (1991) Ten-year results of breast-conserving surgery and definitive irradiation for intraductal carcinoma (ductal carcinoma in situ) of the breast. *Cancer*, **68**, 2337-2344.
19. Lakhani, S.R., Collins N., Sloane J.P. and Stratton M.R. (1995) Loss of heterozygosity in lobular carcinoma in situ of the breast. *J Clin Pathol: Mol Pathol*, **48**, M74-M78.
20. Page, D. and Dupont W. (1992) Benign breast disease: indicators of increased breast cancer risk. *Cancer Detection & Prevention*, **16**, 93-97.

21. Newman, B., Austin M., Lee M. and King M.-C. (1988) Inheritance of human breast cancer: evidence for autosomal dominant transmission in high-risk families. *Proc. Natl. Acad. Sci. USA*, **85**, 3044-3048.
22. Claus, E.B., Risch N. and Thompson W.D. (1991) Genetic analysis of breast cancer in the cancer and steroid hormone study. *Am J Hum Genet*, **48**, 232-242.
23. Anderson, D. (1991) Familial versus sporadic breast cancer. *Cancer*, **70**, 1740-1746.
24. Liu, B., Parsons R., Papadopoulos N., Nicolaides N.C., Lynch H.T., Watson P., Jass J.R., Dunlop M., Wyllie A., Peltomaki P., de la Chapelle A., Hamilton S.R., Vogelstein B. and Kinzler K.W. (1996) Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients [see comments]. *Nat Med*, **2**, 169-74.
25. Kolodner, R., Hall N., Lipford J., Kane M., Rao M., Morrison P., Wirth L. and al e. (1994) Structure of the human MHS2 locus and analysis of two Muir-Torre kindreds for MSH2 mutations. *Genomics*, **24**, 516.
26. Bowcock, A.M., Anderson L.A., Friedman L.S., Black D.M., Osborne-Lawrence S., Rowell S.E., Hall J.M., Solomon E. and King M.C. (1993) THRA1 and D17S183 flank an interval of <4cM for the breast-ovarian cancer gene (BRCA1) on chromosome 17q21. *Am J Hum Genet*, **52**, 718-722.
27. Chamberlain, J., Boehnke M., Frank T., Kiousis S., Xu J., Guo S., Hauser E., Norum R., Helmbold E., Markel D., Keshavari S., Jackson C., Calzone K., Garber J., Collins F. and Weber B. (1993) BRCA1 maps proximal to D17S579 on chromosome 17q21 by genetic analysis. *Am J Hum Genet*, **52**, 792-798.

28. Easton, D., Bishop D., Ford D. and Crockford G. (1993) Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. The Breast Cancer Linkage Consortium. *Am J Hum Gene*, **52**, 678-701.
29. Miki, Y., Swensen J., Shattuck-Eidens D., Futreal P., Harshman K., Tavtigian S., Liu Q., Cochran C., Bennett L., Ding W. and al e. (1994) A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science*, **266**, 66-71.
30. Chapman, M. and Verma I. (1996) Transcriptional activation by BRCA1. *Nature*, **382**, 678-679.
31. Cornelisse, C., Cornelis R. and Devilee P. (1996) Genes responsible for familial breast cancer. *Pathology, Research & Practice*, **192**, 684-693.
32. Futreal, P., Söderkvist P., Marks J., Iglehart J., Cochran C., Barrett J. and Wiseman R. (1992) Detection of frequent allelic loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite lenght polymorphisms. *Cancer Res*, **52**, 2624-2627.
33. Cropp, C., Champeme M.-H., Lidereau R. and Callahan R. (1993) Identification of three regions on chromosome 17q in primary human breast carcinoms which are frequently deleted. *Cancer Res*, **53**, 5617-5619.
34. Saito, H., Inazawa J., Saito S., Kasumi F., Koi S., Sagae S., Kudo R., Saito J., Noda K. and Nakamura Y. (1993) Detailed deletion mapping of chromosome 17q in ovarian and breast cancers: 2-cM region on 17q21.3 often and commonly deleted in tumors. *Cancer Res*, **53**, 3382-3385.

35. Devilee, P. and Cornelisse C. (1990) Genetics of human breast cancer. *Cancer Surv*, **9**, 605-630.
36. Chen, Y., Chen C., Riley D., Allred D., Chen P., Von Hoff D., Osborne C. and Lee W. (1995) Aberrant subcellular localization of BRCA1 in breast cancer. *Science*, **270**, 789-791.
37. Jensen, R., Thompson M., Jetton T., Szabo C., van der Meer R., Helou B., Tonick S., Page D., King M. and Holt J. (1996) BRCA1 is secreted and exhibits properties of a granin. *Nat Genet*, **12**, 303-308.
38. Scully, R., Ganeshan S., Brown M., DeCaprio J., Cannistra S., Feunteun J., Schnitt S. and Livingston D. (1996) Localization of BRCA1 in human breast and ovarian cancer cells. *Science*, **272**, 122.
39. Smith, S., Easton D., Evans D. and Ponder B. (1992) Allele losses in the region 17q12-q21 in familial breast and ovarian cancer involve the wild-type chromosome. *Nat Genet*, **2**, 128.
40. Scully, R., Chen J., Plug A., Xiao Y., Weaver D., J F., Ashely T. and Livingston D. (1997) Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell*, **88**, 265-275.
41. Chen, T., Sahin A. and Aldaz C. (1996) Deletion map of chromosome 16q in ductal carcinoma *in situ* of the breast: refining a putative tumor suppressor gene region. *Cancer Res*, **56**, 5605-5609.
42. Gowen, L., Avrutskaya A., Latour A., Koller B. and Leadon S. (1998) BRCA1 required for transcription-coupled repair of oxidative DNA damage. *Science*, **281**, 1009-1012.
43. Wooster, R., Neuhausen S., Mangion J., Quirk Y., Ford D., Collins N., Nguyen K., Seal S., Tran T., Averill D., Fields P., Marshall G., Narod S. and al e. (1994) Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. *Science*, **265**, 2088-2090.

44. Wooster, R., Bignell G., Lancaster J., Swift S., Seal S., Mangion J., Collins N., Gregory S., Gumbs C. and Micklem G. (1995) Identification of the breast cancer susceptibility gene BRCA2. *Nature*, **378**, 789-792.
45. Struewing, J., Abeliovich D., Peretz T., Avishai N., Kaback M., Collins F. and Brody L. (1995) The carrier frequency of the BRCA1 185delAG mutation is approximately 1 percent in Ashkenazi Jewish individuals. *Nature Genet*, **11**, 198-200.
46. Ford, D., Easton D.F., Stratton M., Narod S., Goldgar D., Devilee P., Bishop D.T., Weber B., Lenoir G., Chang-Claude J., Sobol H., Teare M.D., Struewing J., Arason A., Scherneck S., Peto J., Rebbeck T.R., Tonin P., Neuhausen S., Barkardottir R., Eyfjord J., Lynch H., Ponder B.A., Gayther S.A., Zelada-Hedman M. and et al. (1998) Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. *American J. Human Genetics*, **62**, 676-689.
47. Malkin, D., Li F., Strong L., Fraumeni J.J., Nelson C., Kim D., Kassel J., Gryka M., Bischoff F., Tainsky M. and al e. (1990) Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science*, **250**, 1233-1238.
48. Srivastava, S., Zou Z., Pirollo K., Blattner W. and Chang E. (1990) Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-fraumeni syndrome. *Nature*, **348**, 747-749.
49. Osborne, R., Merlo G., Mitsudomi T., Venesio T., Liscia D., Cappa A., Chiba I., Takahashi T., Nau M., Callahan R. and Minna J. (1991) Mutations in the p53 gene in primary human breast cancers. *Cancer Res*, **51**, 6194-6198.

50. Nelen, M., Padberg G., Peeters E., Lin A., van den Helm B., Frants R., Coulon V., Goldstein A., van Reen M., Easton D., Eeles R., Hodgsen S., Mulvihill J., Murday V., Tucker M., Mariman E., Starink T., Ponder B., Ropers H., Kremer H., Longy M. and Eng C. (1996) Localization of the gene for Cowden disease to chromosome 10q22-23. *Nature Genetics*, **13**, 114-116.
51. Liaw, D., Marsh D., Li J., Dahia P., Wang S., Zheng Z., Bose S., Cell K., Tsou H., Peacocke M., Eng C. and Parsons R. (1997) Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nature Genetics*, **16**, 64-67.
52. Steck, P., Pershouse M., Jasser S., Yung W., Lin H., Ligon A., Langford L., Baumgard M., Hattier T., Davis T., Frye C., Hu R., Swedlund B., Teng D. and Tavtigian S. (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nature Genet*, **15**, 356-362.
53. Li, J., Yen C., Liaw D., Podsypania K., Bose S., Wang S., Puc J., Miliaresis C., Rodgers L., McCombie R., Bigner S., Giovanella B., Ittmann M., Tycko B., Hibshoosh H., Wigler M. and Parsons R. (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science*, **275**, 1876-1878.
54. Cairns, P., Okami K., Halachmi S., Halachmi N., Esteller M., Herman J., Jen J., Isaacs W., Bova G. and Sidransky D. (1997) Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. *Cancer Res*, **57**, 4997-5000.
55. Risinger, J., Hayes A., Berchuck A. and Barrett J. (1997) PTEN/MMAC1 mutations in endometrial cancers. *Cancer Res*, **57**, 4736-4738.

56. Rhei, E., Kang L., Bogomolniy F., Federici M., Borgen P. and Boyd J. (1997) Mutation analysis of the putative tumor suppressor gene PTEN/MMAC1 in primary breast carcinoma. *Cancer Res*, **57**, 3657-3659.
57. Ueda, K., Nishijima M., Inui H., Watatani M., Yayoi E., Okamura J., Yasutomi M., Nakamura Y. and Miyoshi Y. (1998) Infrequent mutations in the PTEN/MMAC1 gene among primary breast cancers. *Japanese J Cancer Res*, **89**, 17-21.
58. Gatti, R., Berkel I., Boder E., Braedt G., Charmley P., Concannon P., Ersoy F., Foroud T., Jaspers N., Lange K., Lathrop G., Leppert M., Nakamura Y., O'Connell P., Paterson M., Salser W., Sanal W., Silver J., Sparkes R., Susi E., Weeks D., Wei S., White R. and Yoder F. (1988) Localization of an ataxia-telangiectasia gene to chromosome 11q22-23. *Nature*, **336**, 577-580.
59. FitzGerald, M., Bean J., Hegde S., Unsal H., MacDonald D., Harkin D., Finkelstein D., Isselbacher K. and Haber D. (1997) Heterozygous ATM mutations do not contribute to early onset of breast cancer. *Nature Genetics*, **15**, 307-310.
60. Chen, J., Birkholtz G., Lindblom P., Rubio C. and Lindblom A. (1998) The role of ataxia-telangiectasia heterozygotes in familial breast cancer. *Cancer Res*, **58**, 1376-1379.
61. Krontiris, T., Devlin B., Karp D., Robert N. and Risch N. (1993) An association between the risk of cancer and mutations in the HRAS1 minisatellite locus. *N Engl. J. Med.*, **329**, 517-523.
62. Garrett, P., Hulka B., Kim Y. and Farber R. (1993) HRAS protooncogene polymorphism and breast cancer. *Cancer Epidemiology, Biomarkers & Prevention*, **2**, 131-138.

63. Hall, J., Huey B., Morrow J., Newman B., Lee M., Jones E., Carter C., Buehring G. and King M. (1990) Rare HRAS alleles and susceptibility to human breast cancer. *Genomics*, **6**, 188-191.
64. Rebbeck, T., Couch F., Kant J., Calzone K., Deshano M., Peng Y., Chen K., Garber J. and Weber B. (1996) Genetic heterogeneity in hereditary breast cancer - role of BRCA1 and BRCA2. *Am J Human Genet*, **59**, 547-553.
65. Ambrosone, C.B. and Shields P.G. (1997) Molecular Epidemiology of Breast Cancer. In Aldaz, C.M., Gould, M.N., McLachlan, J. and Slaga, T.J. (eds), *Etiology of Breast and Gynecological Cancers*. Vol. 396. Wiley-Liss, New York, pp. 83-99.
66. Helzlsouer, K., Selmin O., Huang H., Strickland P., Hoffman S., Alberg A., Watson M., Comstock G. and Bell D. (1998) Associate between glutathione S-transferase M1, P1, and T1 genetic polymorphisms and development of breast cancer. *J Natl Cancer Inst*, **90**, 512-518.
67. Brunet, J., Ghadirian P., Rebbeck T., Lerman C., Garber J., Tonin P., Abrahamson J., Foulkes W., Daly M., Wagnercostalas J., Godwin A., Olopade O., Moslehi R., Liede A., Futreal P., Weber B., Lenoir G., Lynch H. and Narod S. (1998) Effect of smoking on breast cancer in carriers of mutant BRCA1 or BRCA2 genes. *J Natl Cancer Inst*, **90**, 761-766.
68. Devilee, P. and Cornelisse C. (1994) Somatic genetic changes in human breast cancer. *Biochimica et Biophysica Acta*, **1198**, 113-130.
69. Thompson, F., Emerson J., Dalton W., Yang J.-M., McGee D., Villar H., Knox S., Massey K., Weinstein R., Bhattacharyya A. and Trent J. (1993) Clonal chromosome abnormalities in human breast carcinomas I.

Twenty-eight cases with primary disease. *Genes, Chromo Cancer*, **7**, 185-193.

70. Dutrillaux, B., Gerbault-Seureau M. and Zafrani B. (1990) Characterization of chromosomal anomalies in human breast cancer. A comparison of 30 paradiploid cases with few chromosome changes. *Cancer Genet Cytogenet*, **49**, 203-217.

71. Pandis, N., Heim S., Bardi G., Idvall I., Mandahl N. and Mitelman F. (1992) Whole-arm t(1;16) and i(1q) as sole anomalies identify gain of 1q as a primary chromosomal abnormality in breast cancer. *Genes Chromosomes Cancer*, **5**, 235-238.

72. Kallioniemi, A., Kallioniemi O.-P., Sudar D., Rutovitz D., Gray J., Waldman F. and Pinkel D. (1992) Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science*, **258**, 818-820.

73. Kallioniemi, A., Kallioniemi O.-P., Piper J., Tanner M., Stokke T., Chen L., Smith H., Pinkel D., Gray J. and Waldman F. (1994) Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. *Proc Natl Acad Sci USA*, **91**, 2156-2160.

74. Gray, J., Collins C., Henderson I., Isola J., Kallioniemi A., Kallioniemi O.-P., Nakamura H., Pinkel D., Stokke T., Tanner M. and Waldman F. (1994) Molecular Cytogenetics of Human Breast Cancer. .

75. Isola, J., Kallioniemi O., Chu L., Fuqua S., Hilsenbeck S., Osborne C. and Waldman F. (1995) Genetic aberrations detected by comparative genomic hybridization predict outcome in node-negative breast cancer. *Am J Path*, **147**, 905-911.

76. Venter, D.J., Tuzi N.L., Kumar S. and Gullick W.J. (1987) Overexpression of the c-erbB-2 oncoprotein in human breast

carcinomas: immunohistological assessment correlates with gene amplification. *Lancet*, **2**, 69-72.

77. Slamon, D.J., Godolphin W., Jones L.A., Holt J.A., Wong S.G., Keith D.E., Levin W.J., Stuart S.G., Udo J., Ullrich A. and et al. (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science*, **244**, 707-712.

78. Berger, M., Locher G., Saurer S., Gullick W., Waterfield M., Groner B. and Hynes N. (1988) Correlation of C-ERBB-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. *Cancer Res*, **48**, 1238-1243.

79. Zhou, D., Battifora H., Yokota J., Yamamoto T. and Cline M. (1987) Association of multiple copies of the c-erbB-2 oncogene with spread of breast cancer. *Cancer Res*, **47**, 6123-6125.

80. Ravdin, P. and Chamness G. (1995) The c-erbB-2 proto-oncogene as a prognostic and predictive marker in breast cancer: a paradigm for the development of other macromolecular markers - a review. *Gene*, **159**, 19-27.

81. Aldaz, C., Chen T., Sahin A., Cunningham J. and Bondy M. (1995) Comparative allelotype of in situ and invasive human breast cancer: High frequency of microsatellite instability in lobular breast carcinomas. *Cancer Res*, **55**, 3976-3981.

82. Muss, H.B., Thor A.D., Berry D.A., Kute T., Liu E.T., Koerner F., Cirrincione C.T., Budman D.R., Wood W.C., Barcos M. and et al. (1994) c-erbB-2 expression and response to adjuvant therapy in women with node- positive early breast cancer [see comments] [published erratum appears in N Engl J Med 1994 Jul 21;331(3):211]. *N Engl J Med*, **330**, 1260-6.

83. Leitzel, K., Teramoto Y., Konrad K., Chinchilli V.M., Volas G., Grossberg H., Harvey H., Demers L. and Lipton A. (1995) Elevated serum c-erbB-2 antigen levels and decreased response to hormone therapy of breast cancer. *J Clin Oncol*, **13**, 1129-1135.
84. Muller, W., Sinn E., Pattengale P., Wallace R. and Leder P. (1988) Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell*, **54**, 105-115.
85. Petit, A., Rak J., Hung M., Rockwell P., Goldstein N., Fendly B. and Kerbel R. (1997) Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol*, **151**, 1523-1530.
86. Wright, M., Grim J., Deshane J., Kim M., Strong T., Siegal G. and Curiel D. (1997) An intracellular anti-erbB-2 single-chain antibody is specifically cytotoxic to human breast carcinoma cells overexpressing erbB-2. *Gene Ther*, **4**, 317-322.
87. Eccles, S., Court W., Box G., Dean C., Melton R. and Springer C. (1994) Regression of established breast carcinoma xenografts with antibody- directed enzyme prodrug therapy against c-erbB2 p185. *Cancer Res*, **54**, 5171-5177.
88. Sandgren, E.P., Luetteke N.C., Palmiter R.D., Brinster R.L. and Lee D.C. (1990) Overexpression of TGF alpha in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. *Cell*, **61**, 1121-1135.
89. Cohen, B.D., Siegall C.B., Bacus S., Foy L., Green J.M., Hellstrom I., Hellstrom K.E. and Fell H.P. (1998) Role of epidermal growth factor

receptor family members in growth and differentiation of breast carcinoma. *Biochem Soc Symp*, **63**, 199-210.

90. Visscher, D., Wallis T., Awussah S., Mohamed A. and Crissman J. (1997) Evaluation of MYC and chromosome 8 copy number in breast carcinoma by interphase cytogenetics. *Genes Chromosomes Cancer*, **18**, 1-7.

91. Chin, L., Liegeois N., DePinho R. and Schreiber-Agus N. (1996) Functional interactions among members of the Myc superfamily and potential relevance to cutaneous growth and development. *J Investig Dermatol Symp Proc*, **1**, 128-135.

92. White, E. (1996) Life, death and the pursuit of apoptosis. *Genes Dev*, **10**, 1-15.

93. Alexandrow, M.G., Kawabata M., Aakre M. and Moses H.L. (1995) Overexpression of the c-Myc oncoprotein blocks the growth-inhibitory response but is required for the mitogenic effects of transforming growth factor beta 1. *Proc Natl Acad Sci U S A*, **92**, 3239-3243.

94. Evan, G. and Littlewood T. (1993) The role of c-myc in cell growth. *Curr Opin Genet Dev*, **3**, 44-49.

95. Shi, Y., Glynn J., Guilbert L., Cotter T., Bissonnette R. and Green D. (1992) Role for c-myc in activation-induced apoptotic cell death in T cell hybridomas. *Science*, **257**, 212-214.

96. Steiner, P., Rudolph B., Muller D. and Eilers M. (1996) The functions of Myc in cell cycle progression and apoptosis. *Prog Cell Cycle Res*, **2**, 73-82.

97. Leder, A., Pattengale P., Kuo A., Stewart T. and Leder P. (1986) Consequences of widespread deregulation of the c-myc gene in

transgenic mice: multiple neoplasms and normal development. *Cell*, **45**, 485-495.

98. Varley, J., Swallow J., Brammar W., Whittaker J. and Walker R. (1987) Alterations to either C-ERBB-2 (NEU) or C-MYC proto-oncogenes in breast carcinomas correlate with poor short-term prognosis. *Oncogene*, **1**, 423-430.

99. Shiu, R., Watson P. and Dubik (1993) c-myc oncogene expression in estrogen-dependent and independent breast cancer. *Clin Chem*, **39**, 353-355.

100. Lammie, G. and Peters G. (1991) Chromosome 11q13 abnormalities in human cancer. *Cancer Cells*, **3**, 413-420.

101. Gillett, C., Fantl V., Smith R., Fisher C., Bartek J., Dickson C., Barnes D. and Peters G. (1994) Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res*, **54**, 1812-1817.

102. Bartkova, J., Lukas J., Muller H., Lutzht D., Strauss M. and Bartek J. (1994) Cyclin D1 protein expression and function in human breast cancer. *Intl J Cancer*, **57**, 353-361.

103. Sicinski, P., JL D., Parker S., Li T., Fazeli A., Gardner H., Haslam S., Bronson R., Elledge S. and Weinberg R. (1995) Cyclin D1 provides a link between development and oncogenes in the retina and breast. *Cell*, **82**, 621-630.

104. Wang, T., Cardiff R., Zukerberg L., Lees E., Arnold A. and Schmidt E. (1994) Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature*, **369**, 669-671.

105. Weinstat-Saslow, D., Merino M., Manrow R., Lawrence J., Bluth R., Wittenbel K., Simpson J., Page D. and Steeg P. (1995) Overexpression

of cyclin D mRNA distinguishes invasive and in situ breast carcinomas from non-malignant lesions. *Nature Med*, **1**, 1257-1260.

106. Muller, W., Lee F., Dickson C., Peters G., Pattengale P. and Leder P. (1990) The int-2 gene product acts as an epithelial growth factor in transgenic mice. *EMBO J*, **9**, 907-913.

107. Anzick, S.L., Kononen J., Walker R.L., Azorsa D.O., Tanner M.M., Guan X.Y., Sauter G., Kallioniemi O.P., Trent J.M. and Meltzer P.S. (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science*, **277**, 965-968.

108. Knudson, A. (1971) Mutation and Cancer: Statistical study of retinoblastoma. *Proc Natl Acad Sci USA*, **68**, 820-823.

109. Comings, D. (1973) A general theory of carcinogenesis. *Proc Natl Acad Sci USA*, **70**, 3324-3328.

110. Goodrich, D. and Lee W.-H. (1993) Molecular characterization of the retinoblastoma susceptibility gene. *Biochim Biophys Acta*, **1155**, 43-61.

111. Cox, L., Chen G. and Lee E.Y.-H.P. (1994) Tumor suppressor genes and their roles in breast cancer. *Breast Cancer Res Treat*, **32**, 19-38.

112. Weber, J. and May P. (1989) Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am J Hum Genet*, **44**, 388-396.

113. Hudson, T.J., Stein L.D., Gerety S.S., Ma J., Castle A.B., Silva J., Slonim D.K., Baptista R., Kruglyak L., Xu S.-H., Hu X., Colbert A.M.E., Rosenberg C., Reeve-Daly M.P. and al e. (1995) An STS-based map of the human genome. *Science*, **270**, 1945-1954.

114. Brenner, A. and Aldaz C. (1995) Chromosome 9p allelic loss and p16/CDKN2 in breast cancer and evidence of p16 inactivation in immortal breast epithelial cells. *Cancer Res*, **55**, 2892-2895.
115. Trent, J., Yang J.-M., Emerson J., Dalton W., McGee D., Massey K., Thompson F. and Villar H. (1993) Clonal chromosome abnormalities in human breast carcinomas II. Thirty-four cases with metastatic disease. *Genes, Chromo Cancer*, **7**, 194-203.
116. Radford, D., Fair K., Phillips N., Ritter J., Steinbrueck T., Holt M. and Donis-Keller H. (1995) Allelotyping of ductal carcinoma in situ of the breast: deletion of loci on 8p, 13q, 16q, 17p and 17q. *Cancer Res*, **55**, 3399-3405.
117. Fujii, H., Marsh C., Cairns P., Sidransky D. and Gabrielson E. (1996) Genetic divergence in the clonal evolution of breast cancer. *Cancer Res*, **56**, 1493-1497.
118. Radford, D., Fair K., Thompson A., Ritter J., Holt M., Steinbrueck T., Wallace M., Wells S.J. and Donis-Keller H. (1993) Allelic loss on a chromosome 17 in ductal carcinoma in situ of the breast. *Cancer Res*, **53**, 2947-2949.
119. Done, S., Arneson N., Ozcelik H., Redston M. and Andrulis I. (1998) p53 mutations in mammary ductal carcinoma in situ but not in epithelial hyperplasias. *Cancer Res*, **58**, 785-789.
120. Ozbun, M. and Butel J. (1995) Tumor suppressor p53 mutations and breast cancer: a critical analysis. *Adv Cancer Res*, **66**, 71-141.
121. Donehower, L., Godley L., Aldaz C., Pyle R., Shi Y., Pinkel D., Gray J., Bradley A., Medina D. and Varmus H. (1995) Deficiency of p53 accelerates mammary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability. *Genes Dev*, **9**, 882-895.

122. Cornelis, R., van Vliet M., Vos C., Cleton-Jansen A.-M., van de Vijver M., Peterse J., Khan P., Borresen A.-L., Cornelisse C. and Devilee P. (1994) Evidence for a gene on 17p13.3, distal to TP53, as a target for allele loss in breast tumors without p53 mutations. *Cancer Res*, **54**, 4200-4206.
123. Hall, J., Lee M., Newman B., Morrow J., Anderson L., Huey B. and King M. (1990) Linkage of early-onset familial breast cancer to chromosome 17q21. *Science*, **250**, 1684-1689.
124. Futreal, P., Liu Q., Shattuck-Eidens D., Cochran C., Harshman K., Tavtigian S., Bennett L., Haugen-Strano A., Swensen J., Miki Y., Eddington K., McClure M., Frye C. and al e. (1994) BRCA1 mutations in primary breast and ovarian carcinomas. *Science*, **266**, 120-122.
125. Leone, A., McBride O., Weston A., Wang M., Anglard P., Cropp C., Goepel J., Lidereau R., Callahan R., Linehan W., Rees R., Harris C., Liotta L. and Steeg P. (1991) Somatic allelic deletion of nm23 in human cancer. *Cancer Res*, **51**, 2490-2493.
126. Cropp, C., Lidereau R., Leone A., Liscia D., Cappa A., Campbell G., Barker E., Le Doussal V., Steeg P. and Callahan R. (1994) NME1 protein expression and loss of heterozygosity mutations in primary human breast tumors. *J Natl Cancer Inst*, **86**, 1167-1169.
127. Borg, A., Zhang Q.-X., Alm P., Olsson H. and Sellberg G. (1992) The retinoblastoma gene in breast cancer: allele loss is not correlated with loss of gene protein expression. *Cancer Res*, **52**, 2991-2994.
128. Miki, Y., Katagiri T., Kasumi F., Yoshimoto T. and Nakamura Y. (1996) Mutation analysis in the BRCA2 gene in primary breast cancers. *Nature Genetics*, **13**, 245-247.

129. Teng, D., Bogden R., Mitchell J., Baumgard M., Bell R., Berry S., Davis T., Ha P., Kehler R., Jammulapati S., Chen Q., Offit K., Skolnick M., Tavtigian S., Jhanwar S., Swedlund B., Wong A. and Kamb A. (1996) Low incidence of BRCA2 mutations in breast carcinoma and other cancers. *Nature Genet*, **13**, 241-244.
130. Lancaster, J.M., Wooster R., Mangion J., Phelan C.M., Cochran C., Gumbs C., Seal S., Barfoot R., Collins N., Bignell G., Patel S., Hamoudi R., Larsson C., Wiseman R.W., Berchuck A., Iglehart J.D., Marks J.R., Ashworth A., Stratton M.R. and Futreal P.A. (1996) BRCA2 mutations in primary breast and ovarian cancers. *Nature Genetics*, **13**, 238-240.
131. Schott, D., Chang J., Deng G., Kurisu W., Kuo W., Gray J. and Smith H. (1994) A candidate tumor suppressor gene in human breast cancers. *Cancer Res*, **54**, 1393-1396.
132. Sato, T., Tanigami A., Yamakawa K., Akiyama F., Kasumi F., Sakamoto G. and Nakamura Y. (1990) Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. *Cancer Res*, **50**, 7184-7189.
133. Tsuda, H., Callen D., Fukutomi T., Nakamura Y. and Hirohashi S. (1994) Allele loss on chromosome 16q24..2-qter occurs frequently in breast cancer irrespectively of differences in phenotype and extent of spread. *Cancer Res*, **54**, 513-517.
134. Cleton-Jansen, A., Moerland E., Kuipers-Dijkshoorn N., Callen D., Sutherland G., Hansen B., Devilee P. and Cornelisse C. (1994) At least two different regions are involved in allelic imbalance on chromosome arm 16q in breast cancer. *Genes, Chromos & Cancer*, **9**, 101-107.

135. Berx, G., Cleton-Jansen A., Nollet F., de Leeuw W., van de Vijver M., Cornelisse C. and van Roy F. (1995) E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. *Embo J*, **14**, 6107-6115.
136. Yoshiura, K., Kanai Y., Ochiai A., Shimoyama Y., Sugimura T. and Hirohashi S. (1995) Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. *Proc Natl Acad Sci USA*, **92**, 7416-7419.
137. Rimm, D., Sinard J. and Morrow J. (1995) Reduced  $\alpha$ -catenin and E-cadherin expression in breast cancer. *Lab Invest*, **72**, 506-512.
138. Lee, S. (1996) H-cadherin, a novel cadherin with growth inhibitory functions and diminished expression in human breast cancer. *Nature Medicine*, **2**, 776-782.
139. Kamb, A., Gruis N., Weaver-Feldhaus J., Qingyun L., Harshman K., Tavtigian S., Stockert E., Day R., Johnson B. and Skolnick M. (1994) A cell cycle regulator potentially involved in genesis of many tumor types. *Science*, **264**, 436-440.
140. Brenner, A., Paladugu A., Wang H., Olopade O., Dreyling M. and Aldaz C. (1996) Preferential loss of expression of p16<sup>INK4a</sup> rather than p19<sup>ARF</sup> in breast cancer. *Clinical Cancer Res*, **2**, 1993-1998.
141. Altucci, L., Addeo R. and Cicatiello L. (1996) 17b-estradiol induces cyclin D1 gene transcription, p36D1-p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells. *Oncogene*, **12**, 2315-2324.
142. Sherr, C. (1994) G1 phase progression: cycling on cue. *Cell*, **79**, 551-555.
143. Sherr, C. (1996) Cancer cell cycles. *Science*, **274**, 1672-1677.

144. Hui, R., Cornish A., McClelland R., Robertson J., Blamey R., Musgrove E., Nicholson R. and Sutherland R. (1996) Cyclin D1 and estrogen receptor messenger RNA levels are positively correlated in primary breast cancer. *Clin Cancer Res*, **2**, 923-928.
145. Graybablin, J., Zalvide J., Fox M., Kinckerbocker C., Decaprio J. and Keyomarsi K. (1996) Cyclin E, a redundant cyclin in breast cancer. *Proc Natl Acad Sci USA*, **93**, 15215-15220.
146. Porter, P., Malone K., Heagerty P., Alexander G., Gatti L., Firpo E., Daling J. and Roberts J. (1997) Expression of cell-cycle regulators p27<sup>Kip1</sup> and cyclin E, alone and in combination, correlate with survival in young breast cancer patients [see comments]. *Nat Med*, **3**, 222-225.
147. Varley, J., Armour J., Swallow J., Jeffreys A., Ponder B., T'Ang A., Fung Y., Brammar W. and Walker R. (1989) The retinoblastoma gene is frequently altered leading to loss of expression in primary breast tumours. *Oncogene*, **4**, 725-729.
148. Okamoto, A., Demetrick D., Spillare E., Hagiwara K., Hussain S., Bennett W., Forrester K., Gerwin B., Serrano M., Beach D. and Harris C. (1994) Mutations and altered expression of p16INK4 in human cancer. *Proc Natl Acad*, **91**, 11045-11049.
149. Parry, D., Bates S., Mann D. and Peters G. (1995) Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of p16INK4/MTS1 tumour suppressor product. *EMBO J*, **14**, 503-511.
150. Xiong, Y., Hannon G., Zhang H., Casso D., Kobayashi R. and Beach D. (1993) p21 is a universal inhibitor of cyclin kinases. *Nature*, **366**, 701-704.

151. Waga, S., Hannon G., Beach D. and Stillman B. (1994) The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature*, **369**, 574-578.
152. Brugarolas, J., Chandrasekaran C., Gordon J., Beach D., Jacks T. and Hannon G. (1995) Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature*, **377**, 552-557.
153. Pomerantz, J., Schreiber-Agus N., Liegeois N., Silverman A., Alland L., Chin L., Potes J., Chen K., Orlow I., Lee H.-W., Cordon-Cardo C. and DePinho R. (1998) The Ink4a tumor suppressor gene product, p19<sup>Arf</sup>, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell*, **92**, 713-723.
154. Zhang, Y., Xiong Y. and Yarbrough W.G. (1998) ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell*, **92**, 725-734.
155. Bates, S., Phillips A.C., Clark P.A., Stott F., Peters G., Ludwig R.L. and Vousden K.H. (1998) p14ARF links the tumour suppressors RB and p53. *Nature*, **395**, 124-125.
156. Oltvai, Z., Milliman C. and Korsmeyer S. (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, accelerates programmed cell death. *Cell*, **74**, 609-619.
157. Joensuu, H., Pylkkanen L. and Toikkanen S. (1994) Bcl-2 protein expression and long-term survival in breast cancer. *Am J Pathol*, **145**, 1191-1198.
158. Barbareschi, M., Caffo O., Veronese S., Leek R., Fina P., Fox S., Bonzanini M., Girlando S., Morelli L., Eccher C., Pezzella F., Doglioni C., Dalla Palma P. and Harris A. (1996) Bcl-2 and p53 expression in

node-negative breast carcinoma: a study with long-term follow-up.  
*Human Pathol.*, **27**, 1149-1155.

159. Sierra, A., Castellsague X., Coll T., Manas S., Escobedo A., Moreno A. and Fabra A. (1998) Expression of death-related genes and their relationship to loss of apoptosis in T1 ductal breast carcinomas. *Intrnl. J. Cancer*, **79**, 103-110.

160. Beatson, G. (1896) On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. *Lancet*, **ii**, 104-107.

161. Fishman, J., Osborne M. and Telang N. (1995) The role of estrogen in mammary carcinogenesis. *Annals NY Academy of Sciences*, **768**, 91-100.

162. Tsai, M. and O'Malley B. (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Ann Rev Biochem*, **63**, 451-486.

163. Leung, B. and Potter A. (1987) Mode of estrogen action on cell proliferation in CAMA-1 cells: II. Sensitivity of G1 phase population. *J Cellular Biochem*, **34**, 213-225.

164. Davidson, N., Prestigiacomo L. and Hahn H. (1993) Induction of jun gene family members by transforming growth factor alpha but not 17 beta-estradiol in human breast cancer cells. *Cancer Research*, **53**, 291-297.

165. Prall, O., Sarcevic B., Musgrove E., Watts C. and Sutherland R. (1997) Estrogen-induced activation of Cdk4 and Cdk2 during G1-Sphase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk2. *J Biol Chem*, **272**, 10882-10894.

166. Zwijsen, R., Wientjens E., Klompmaker R., van der Sman J., Bernards R. and Michalides R. (1997) CDK-independent activation of estrogen receptor by cyclin D1. *Cell*, **88**, 405-415.
167. Zhang, L., Zhou W., Velculescu V., Kern S., Hruban R., Hamilton S., Vogelstein B. and Kinzler K. (1997) Gene expression profiles in normal and cancer cells. *Science*, **276**, 1268-1272.

## **Figure Legend**

**Figure 1.** Diagrammatic depiction of the multiple endogenous and exogenous factors which contribute to breast cancer risk. Ultimately, it is the combination of multiple factors along with the unique genetic composition of each individual woman that plays a decisive role in defining the risk for tumor development. This etiologic complexity is also responsible for the characteristic heterogeneity of breast cancer.

**Figure 2.** A diagrammatic representation of the normal breast structure and histology (modified from Tavassoli 1992).

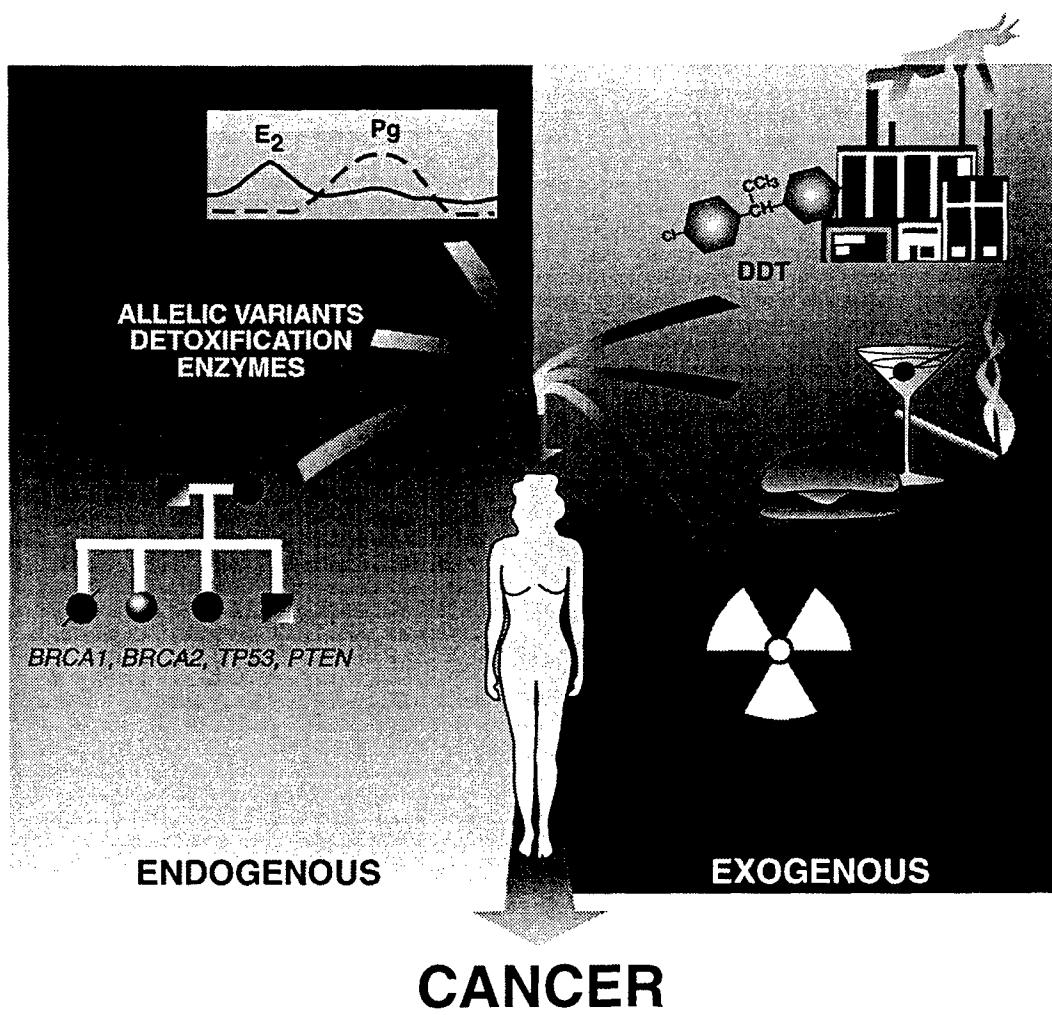
**Figure 3.** A graphic depiction of the 'sporadic' breast cancer incidence as compared to hereditary breast cancer incidence.

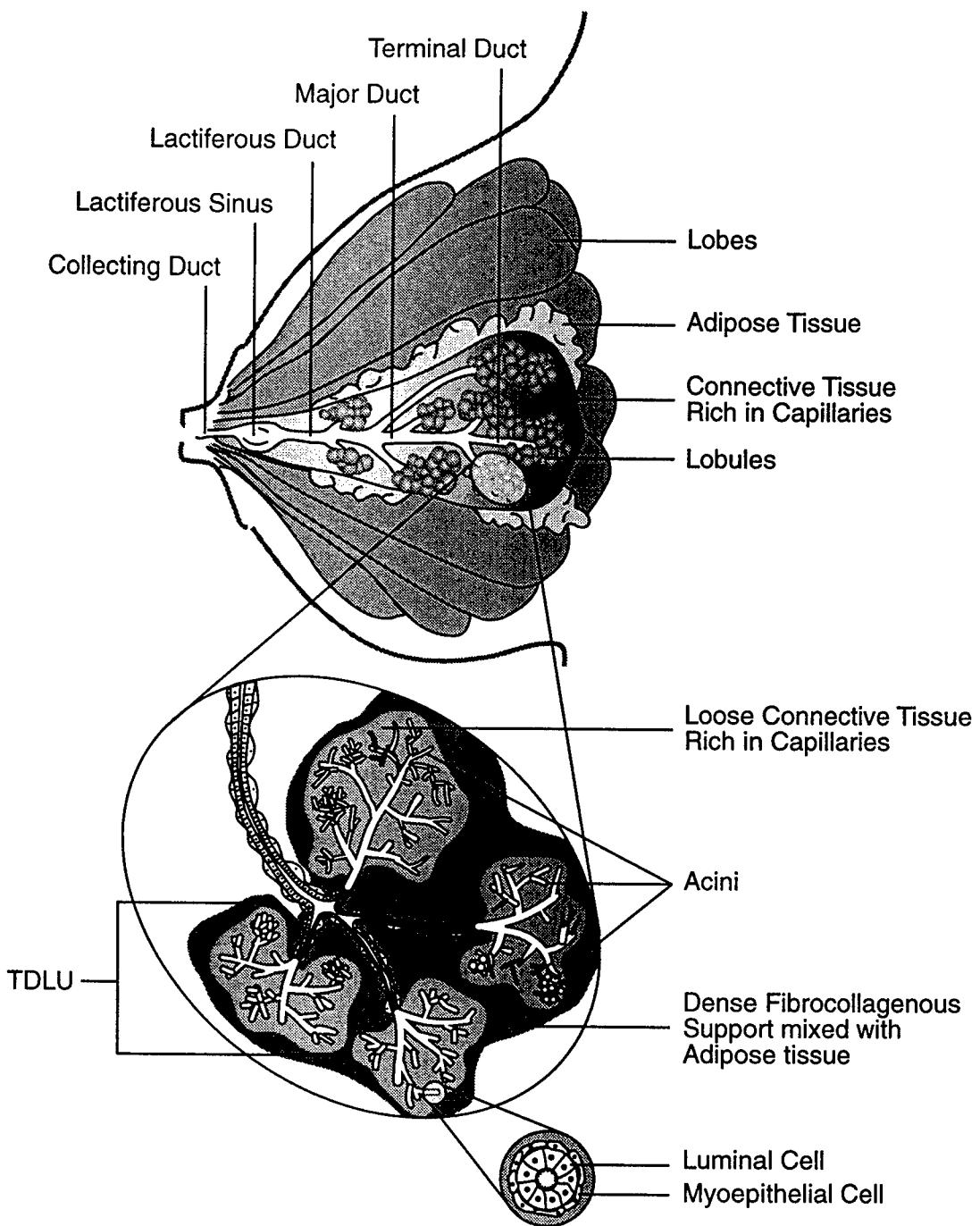
**Figure 4.** Schematic putative model of breast cancer histopathological progression and corresponding estimated breast cancer risk based on studies by Page and Dupont (Page DL, Dupont WD, 1992). A woman with PDWA (proliferative disease without atypia) has a 1.5 to 2 times greater risk for developing breast cancer as compared to the general population. Whereas, a women with atypical hyperplasia (AH) has 4-5 times greater risk. Women with DCIS (ductal carcinoma in situ) and LCIS (lobular carcinoma in situ) are at a much higher risk of progressing to invasive cancer. LNG is low nuclear grade and HNG is high nuclear grade

**Figure 5.** Schematic representation of cell cycle's G1/S key restriction point controls many of which are known to be altered in breast cancer (see text).

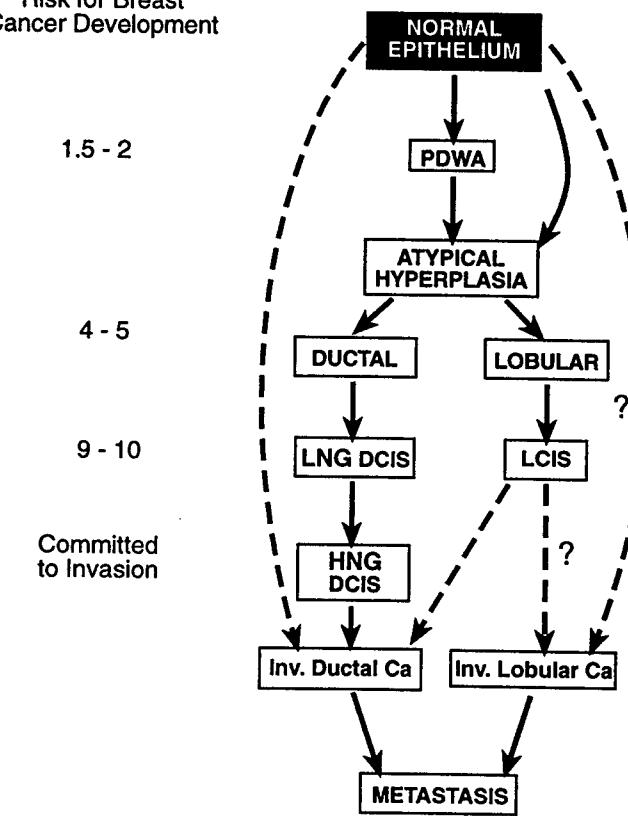
Genetic Region	Cytogenetic Finding	CGH <sup>2</sup> Finding	Invasive <sup>3</sup> LOH (%)	DCIS <sup>3</sup> LOH (%)	Possible Targets	Possible Consequence
1p	-1p		32	8		
1q	+1q	+1q	30	16		
3p		-3p	22	0		
3q			25	0		
4p	-4p		2 <sup>1</sup>	0 <sup>†</sup>		
5p			18 <sup>1</sup>	0 <sup>†</sup>		
5q			13 <sup>1</sup>	0 <sup>†</sup>		
6p	-6	+6p	30	0		
6q	-6, -6q	-6q, +6q	26	8		
7p	+7	+7p	32	32	<i>EGFR</i>	overexpression
7q	+7		25	24		
8p	-8, -8p	-8p	18	10		
8q	-8, +8q	+8q	20	22	<i>MYC</i>	overexpression
9p	-9p		58	30	<i>p16INK4a</i>	↓ expression/ dysregulation
9q			24 <sup>1</sup>	0 <sup>†</sup>		
10p			11 <sup>1</sup>	0 <sup>†</sup>		
10q			15 <sup>1</sup>	0 <sup>†</sup>		
11p	-11, -11p	-11p	28	0		
11q	-11, -11q	+11q	30	12	<i>CCND1 (cyclin D1)</i>	overexpression
12p			8 <sup>1</sup>	0 <sup>†</sup>		
12q		+12q	4 <sup>1</sup>	0 <sup>†</sup>		
13q	-13	-13q	30	18	<i>RB1, BRCA2, Brush</i>	↓ expression
16p	-16		40	0		
16q	-16, -16q		48	27	<i>CDH1 (E. cadherin)</i>	↓ expression
17p	-17, -17p	-17p	57	33	<i>TP53</i>	inactivation
17q	-17		36	31	<i>BRCA1, NME1</i>	↓ expression
		+17q			<i>ERBB2</i>	overexpression
18p			25	0		
18q			48	12	<i>BCL2</i>	dysregulation
19p			18 <sup>1</sup>	0 <sup>†</sup>		
19q		+19q	14 <sup>1</sup>	0 <sup>†</sup>	<i>CCNE (cyclin E)</i>	overexpression
20q		+20q13	17 <sup>1</sup>	6 <sup>†</sup>	<i>AIB1</i>	overexpression
21q			17 <sup>1</sup>	5 <sup>†</sup>		
22q	-22		36	0		
Xp	-X		22 <sup>1</sup>			
Xq	-X		8 <sup>1</sup>			

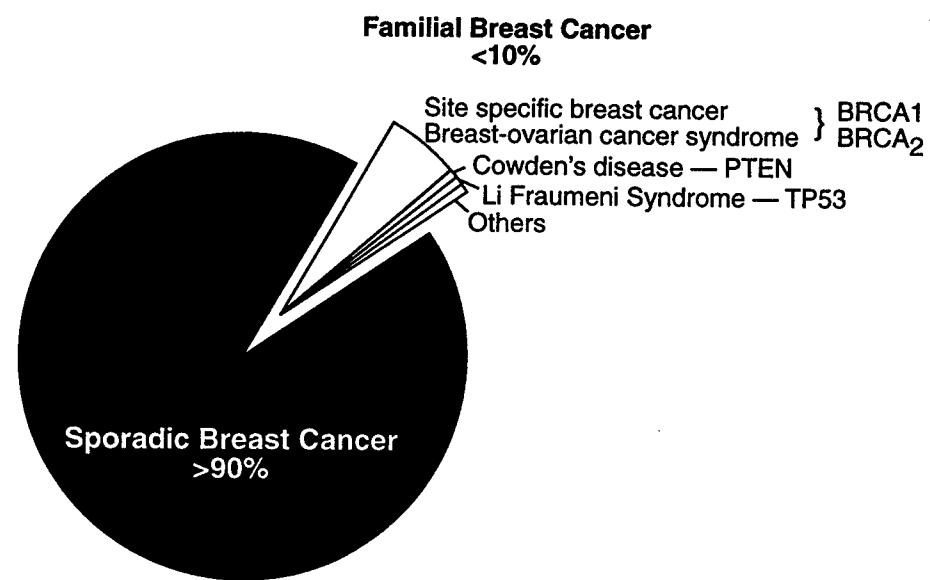
Table 1. Summary of genetic aberrations affecting sporadic breast cancer (<sup>1</sup> Devilee and Cornelisse [1994], Thompson et. al. [1993]; <sup>2</sup> Gray et. al. [1994]; <sup>3</sup> Aldaz et. al. [1995]; <sup>†</sup> Radford et. al. [1995]).

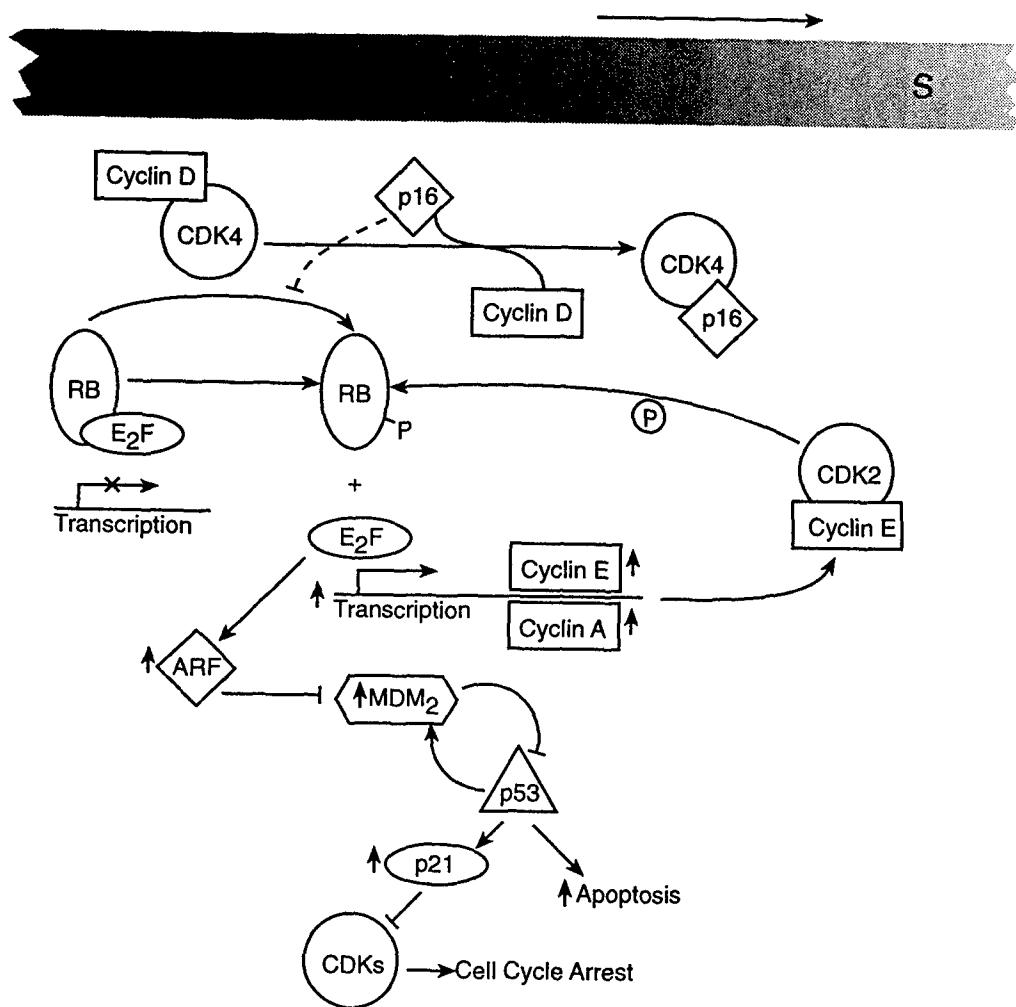




Risk for Breast  
Cancer Development







CHROMOSOME 9P LOH AND ANALYSIS OF MTS1 (P16) IN BREAST CANCER. Brenner AJ\* and Aldaz CM. The University of Texas M.D. Anderson Cancer Center, Science Park - Research Division, Smithville, Texas 78957

In order to define the extent of involvement of chromosome 9p in breast carcinogenesis, we performed microsatellite length polymorphism analysis of markers spanning this region. Of 24 primary breast carcinomas analyzed, we observed a high frequency (58%) of loss of heterozygosity (LOH) or allelic imbalance affecting subregion 9p21-22. Mutational analysis of the CDKN2 (p16) tumor suppressor gene was performed to determine whether this gene was the target of such alterations. Of 21 tumors analyzed, only 1 showed a mutation of probable consequence, suggesting that CDKN2 appears not to be the target of LOH. However, gene inactivation *in vivo* has not yet been explored, and we are currently investigating this prospect. Additionally, since it has been suggested that some CDKN2 deletions and mutations could be due to an *in vitro* phenomenon, 4 immortal breast cell lines derived from normal epithelium, MCF10F, MCF12F, 184A1, and 184B5, were examined for loss or mutation of CDKN2. Two lines (MCF10F and MCF12F) showed homozygous deletions of CDKN2, and one (184A1) revealed a hemizygous deletion and a nonsense mutation in the remaining allele. This could imply an important role of CDKN2 in the control of immortalization or *in vitro* adaptation, and is the first evidence of such in non-tumor derived cell lines. Additionally, this is the first report of frequent LOH in the 9p21-22 chromosome subregion of uncultured primary breast tumors. We are currently investigating the growth suppression potential of p16 in the aforementioned lines as well as the expression of p16 *in vivo*. Supported by U.S. Army Brest Cancer Program, DAMD 17-94-J-4078 (CMA) and NIH R01 CA59967 (CMA).

## CARCINOGENESIS

702

**Allelotype of breast carcinoma *in situ* from paraffin sections, correlation with chromosome copy number.**  
Chen, T., Dhingra, K., Brenner, A., Sahin, A., Sniege, N., Vogel, V., Hortobagyi, G.N., & Aldaz, C.M. Dept. of Carcinogenesis, Dept. of Breast and Gynecologic Oncology, Dept. of Pathology, Dept. of Medical Oncology, University of Texas M.D. Anderson Cancer Center, Smithville, TX 78957 and Houston, TX, 77030.

We have optimized a technique that allows the analysis of numerous chromosomal loci from single 8  $\mu$ m sections of paraffin embedded tissues. DNA samples from normal and breast CIS tissue can be obtained from the same section by means of microdissection. Further improvement was achieved by the use of a short PCR round with a universal primer followed by the use of the specific microsatellite primers. Numerous markers were analyzed spanning the 17 p and q arms. Higher incidence of losses was observed affecting the p arm, close to the TP53 locus. The same samples were analyzed for ch 17 copy number by means of *in situ* hybridization. Analysis of the same samples is being extended to all the chromosome areas of interest in breast cancer and correlations will be performed with multiple diagnostic/prognostic indicators. Supported by NIH grant CA58186.

703

**Microsatellite instability in Muir-Torre syndrome.**  
Halling, K.C., Honchel, R., Piteikow, M., and Thibodeau, S.N. Mayo Clinic and Foundation, Rochester, MN 55905

Muir-Torre syndrome (MTS) is characterized by at least one sebaceous gland tumor and at least one visceral malignancy. Although a wide range of internal malignancies have been reported, the most frequently observed internal neoplasm is colorectal carcinoma. MTS and hereditary non-polyposis colorectal carcinoma (HNPCC) share many clinical and pathologic characteristics, and thus may share similar genetic mechanisms of tumorigenesis. Microsatellite instability (MI) has recently been reported in tumor tissue from HNPCC patients. In order to determine if tumors from MTS patients might also show MI, we examined DNA extracted from paraffin-embedded tissues for the presence of MI at (CA)<sub>n</sub> repeats on chromosomes 5q, 15q, 17p, and 18q. Data was obtained on 12 patients diagnosed with MTS, 9 of which had at least one colorectal tumor. Of the 12 MTS patients, six had widespread MI in all sebaceous and colorectal tumors examined, as well as in a prostatic adenocarcinoma, a transitional cell carcinoma of the bladder and two keratoacanthomas. These patients were characterized by: 1) uniform presence and early onset of colorectal cancer (average age of 39.7 yrs; range 23-53); and 2) prolonged survival following diagnosis of colorectal cancer (average survival of 30.5 yrs; range 23-37). These results indicate that MTS and HNPCC may share similar genetic mechanisms of tumorigenesis, and supports the notion that these syndromes are allelic.

704

**(CA)<sub>n</sub> repeat alterations in human gastric adenocarcinomas and hepatocellular carcinomas (HCC).**  
Mironov, N.M., Aguelon, A.-M., Potapova<sup>1</sup>, G.I., Krutovskikh, V.A., Mazzoleni, G., Omori, Y., Gorbunov<sup>1</sup>, O.V., Klimenkov<sup>1</sup>, A.A. & Yamasaki, H. International Agency for Research on Cancer, Lyon, France, and <sup>1</sup>Cancer Research Center, Moscow, Russia.

The human genome contains approximately 50,000-100,000 loci of (CA)<sub>n</sub> repeats. Ten of these were chosen at random and two distinct patterns of alteration were identified in human tumors: loss of heterozygosity (LOH) and gain of new (shorter or longer) repeats. Among 15 gastric adenocarcinomas, one tumor had LOH at 5 loci out of the 10 studied, 3 gained a new sequence at 1 locus and one showed gains at 5 loci. Three tumors out of 15 carried mutations in the p53 gene, but only one showed (CA)<sub>n</sub> repeat alterations (LOH) and no mutation was found in the connexin 32 gene. This indicates that genomic instability at (CA)<sub>n</sub> sites does not confer an ability to produce abundant point mutations. In contrast, among 11 human HCCs studied, only one had LOH at 1 locus and no gain of new repeats. Our results suggest that different mechanisms exist for LOH and gain of new (CA)<sub>n</sub> repeats, and their prevalence differs between human gastric and liver cancer.

705

**Chromosomal changes during the acquisition of doxorubicin resistance in human leukemia HL-60 cells.** R. Ganapathi, G. Hoeltge, D. Grabowski, R. Neelon, and J. Ford. Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195.

Tumor cell resistance to doxorubicin (DOX) is usually associated with the overexpression of P-glycoprotein (PGP) in model systems. We have characterized the karyotypic changes in two sublines of HL-60 cells designated HL-60/A and HL-60/Y, selected in increasing concentrations of 0.025-0.1  $\mu$ g/ml DOX. Monosomy 8 in HL-60/Y was the only karyotypic difference prior to DOX exposure. Both sublines acquired 7q+ markers upon exposure to DOX. In HL-60/Y, an add(7)(q21) replaced one homologue at 0.025  $\mu$ g/ml DOX, and an add(7)(q32) appeared which replaced the other normal 7 at 0.05  $\mu$ g/ml DOX. The HL-60/A cells acquired an add(7)(q31) at 0.025  $\mu$ g/ml DOX. Exact mechanisms of these 7q+ abnormalities could not be determined, but all involved breakpoints in the midregion of 7q. The overexpression of PGP in immunoprecipitates with C-219 antibody in both sublines of DOX-resistant HL-60 cells with 7q+ abnormalities is consistent with the location of MDR1 sequences to 7q21.1. (Supported by USPHS CA35531).

706

**Hodgkin and Reed-Sternberg cells do not show lymphoid differentiation: Results of single cell analysis at the DNA level**

Daus, H., Trümper, L., Roth, J., Jacobs, G., v. Bonin, F., Gause, A., Pfreundschuh, M. Dep. Internal Medicine I, University of Saarland at 66421 Homburg/Saar, Germany

Investigations on the nature of Hodgkin's disease have been hampered by the fact that the neoplastic Hodgkin and Reed-Sternberg (H&RS) cells represent only a minority of the cells in affected tissue. Since a lymphoid origin of H&RS cells has been proposed, and IgH and T-cell receptor gamma chain rearrangements can serve as markers for origin and clonality in B and T lymphocytes, we developed a PCR based assay to detect IgH or TCR rearrangements in single H&RS cells. Single cells were isolated by micromanipulation from cytopsins prepared from fresh HD lymph nodes. Primers were constructed corresponding to the variable and joining regions of the IgH and TCR-gamma genes and for detection of N-RAS codon 12/13 or 61 Mutations. Oligo hybridization and SSPC/direct sequencing were used to confirm the presence of rearrangements or mutations. These methods were established on single cells of different lymphoid cell lines and Non-Hodgkin's lymphomas. Immunoglobulin rearrangements were not detected in H&RS cells of 15/15 patients, T cell receptor gamma rearrangements were absent in 15/15 patients. N-ras codon 12/13 or 61 mutations could not be detected in one case examined so far. These results suggest that H&RS cells are not derived from T or B lineage cells that have initiated DNA rearrangements. The role of N-ras mutations in the pathogenesis of HD remains uncertain.

707

**Microsatellite-based high resolution deletion mapping of chromosome 8p in prostate cancer**

L.Schmidt, R.Leibowitz<sup>1</sup>, K.Tory, M.-H.Wei, H.Li, Peter Scardino<sup>1</sup>, M.Linehan<sup>2</sup> and B.Zbar<sup>3</sup>; PRI/DynCorp, FCRDC, Frederick, MD 21702;

<sup>1</sup>Baylor College of Medicine, Dept. of Pathology, Houston, TX 77030;

<sup>2</sup>Surgery Br., NCI, Bethesda, MD 20892; <sup>3</sup>LIB, NCI-FCRDC, Frederick, MD 21702

Previous studies by a number of laboratories show frequent allelic loss on chromosome 8p in several cancer types including bladder, liver, lung, colorectal and prostate (Bova,S. et al, Cancer Res. 53:1-5, 1993). Frequent loss of heterozygosity (LOH) at a chromosomal locus in a particular tumor may indicate the presence of a tumor suppressor gene (TSG). In an effort to localize the site of a putative TSG for prostate cancer, we have examined LOH on chromosome 8p in paired prostate tumor and normal tissue samples. To date twenty pairs have been analysed using eleven chromosome 8 microsatellite markers mainly from Genethon, (Weissenbach,J. et al, Nature 359:794-801, 1992) several RFLP markers and a newly characterized microsatellite marker from our laboratory, located near the MSR gene. These microsatellite markers have heterozygosities >70% and therefore are informative with more than two-thirds of the samples. LOH was observed in 25% of the prostate tumor samples, with the region of minimal deletion lying between D8S265 and D8S261. The high density of the ordered microsatellite markers will allow fine resolution mapping of deletions in prostate tumor samples and more narrowly define the localization of a putative TSG.

## MOLECULAR BIOLOGY/BIOCHEMISTRY

3226

**Defective Mismatch Repair and Microsatellite Instability in Human Cancer Cell Lines.** Boyer, JC, Umar, A, Risinger, J, and Kunkel, TA, Natl. Inst. of Environ. Health Sci., RTP, NC 27709.

Microsatellite instability has been shown to be associated with several types of cancer, both hereditary and sporadic. Although this phenotype has been designated RER+ (for Replication Error), it has not yet been demonstrated to result from a defect in the fidelity of DNA replication. Rather, extracts of several RER+ colon and endometrial cancer lines have been shown to be defective in mismatch repair (MMR). Some of these lines contain mutations in the human homologs of *E. coli* MMR genes, *mutS* (*HMSH2*) or *mutL* (*hMLH1*). Here we have measured MMR activity and RER status of human cell lines derived from tumors of the colon, cervix, endometrium, uterus, and ovary. All that are MMR+ are RER- and all that are MMR- are RER+. This strengthens the correlation between microsatellite instability and defective MMR and suggests that the loss of MMR activity is a step in carcinogenesis common to many types of cancer. Additionally, we have found that human cell extracts are proficient in repair of heteroduplexes containing loops of five to 16 bases, a repair activity not seen in *E. coli*. This activity is present in an extract of a colon cancer line defective in the *hMLH1* gene product, suggesting that repair of heteroduplexes containing a large loop may have different protein requirements than does repair of mismatches.

3227

**Allelotypic profile and replication error phenotype in lobular vs. ductal and *in situ* breast cancers.** Chen, T., Sahin, A., Aldaz, C.M. UT MD Anderson Cancer Center, Smithville, Texas 78957

We are currently analyzing for specific allelic losses or imbalances and replication error phenotypes (RER) a series of early breast cancer samples from paraffin embedded tissues by means of microsatellite (SSRs) length polymorphisms. In our series of 58 breast cancer samples we analyzed 25 invasive ductal carcinomas, 19 *in situ* ductal carcinomas and 14 invasive lobular carcinomas. The SSRs markers used were representative of the chromosome subregions more commonly involved in abnormalities in breast cancer. We observed allelic imbalances affecting the p and q arms of chr. 1 in approximately 40% of invasive ductal tumors but only in 10-14% of lobular tumors. However these abnormalities appear to be a late event in ductal tumors carcinogenesis since they were not observed at the *in situ* stage. On the other hand abnormalities affecting ch. 11p15.5 appear more common in lobular tumors than in ductal carcinomas. Abnormalities affecting 16q, 17p, 17q appear to be early events since were observed in ~ 30% of carcinomas *in situ*. Interestingly the RER+ phenotype was more frequent in lobular tumors since we observed microsatellite instability affecting various SSRs in 5 out 14 tumors (36%) vs. 12% and 16% for invasive and *in situ* ductal tumors respectively. Our data suggests that lobular breast carcinomas appear as a different entity when compare with ductal tumors possibly involving different mechanisms of carcinogenesis. (U.S. Army Grant DAMD17-94-J-4078)

3228

**Genomic instability in mouse mammary tumors with p53 deficiency or mutation.** Aldaz, C.M.<sup>1</sup>, Paladugu, A.<sup>1</sup>, Medina, D.<sup>2</sup>, Donehower, L.A.<sup>2</sup>

<sup>1</sup>University of Texas M.D. Anderson Cancer Center, Smithville, Texas 78957;

<sup>2</sup>Baylor College of Medicine, Houston, Texas 77030

We analyzed the karyotypes of mammary tumors generated in *Wnt-1* transgenic mice lacking one or both p53 germline alleles. Tumors from p53<sup>+/+</sup> animals were mostly diploid while tumors from p53<sup>-/-</sup> animals presented highly abnormal karyotypes. As a cytogenetic measure of gene amplification and instability we quantified the percentage of metaphases per tumor with homogeneously staining regions (HSR), dicentric chromosomes or double minutes. All mammary tumors (n = 6) from p53<sup>-/-</sup> animals showed these abnormalities, ranging from 17% to 62% metaphases/tumor with these anomalies;  $\bar{x}$  for this group = 40% vs. 3% for the tumors from p53<sup>+/+</sup> mice (n = 5). Using a different mouse model we analyzed the karyotypes of tumors derived from transplantable mammary (TM) hyperplastic outgrowth lines (Kittrell et al. Cancer Res. 52:1924, 1992). TM lines and derived tumors were shown to have p53 inactivating mutations or mutations leading to p53 overexpression (Jerry et al. Cancer Res. 53:3374, 1993). Seven tumors were analyzed from 3 different TM lines. All the tumors showed highly abnormal and unstable karyotypes, six of the tumors showed multiple chromosomes with HSRs. The only consistent feature observed in 5 of 7 tumors was overrepresentation of chromosome 6. These studies with two *in vivo* tumorigenesis models support the hypothesis of p53 as a major player in maintaining genomic stability. Supported by NIH CA59967.

3229

**4-nitroquinoline-1-oxide (4NQO) induced oral cavity Ha-ras mutations lead to loss of heterozygosity (LOH), gene amplification, and genomic instability during neoplastic transformation.** L. Hu, B. Yuan, F. Hender. Depts of Biochemistry & Medicine, Henry Vogt Cancer Institute, University of Louisville, and Louisville VAMC, Louisville, KY 40292.

To study the molecular events involved in human head and neck squamous cell carcinoma (HNSCC), we have developed a murine model using 4NQO induced carcinogenesis. In 13 of 25 HNSCC 4NQO induced Ha-ras mutations at codon 12 (Yuan et al. Cancer Res., 1994). Seven HNSCC had lost both normal Ha-ras alleles. Microsatellite markers identified LOH which always occurred on chromosome (chr) 7-telomeric to the tyrosinase gene. There was no evidence of trisomy. Quantitative PCR demonstrated that 3 of 5 tumors with LOH had evidence of up to 5 fold *int-2* amplification. Genomic instability was determined by comparing DNA fingerprints of germline and tumor tissue amplified by PCR with arbitrary primers having less than 50% homology with any known gene. All 3 tumors with *int-2* amplification had widespread genomic instability. FISH analysis demonstrated that the genomic instability involved chromosomes in addition to chr 7. No *int-2* amplification or genomic instability was detected in tumors without LOH at the Ha-ras locus on chr 7. Since these molecular events occurred at least 5 months after 4NQO had ceased, this suggests that the initial Ha-ras mutation is associated with a cascade of molecular events during tumorigenesis that eventuates in LOH and genomic instability in the absence of further carcinogenic pressure.

3230

**Early apoptotic cell death and regenerative proliferation in livers of folate/methyl deficient rats is accompanied by nucleotide depletion and increased DNA repair activity.** James, S.J., Pogribny, I., Basnakian, A.G., Miller, B.J., and Muskhelishvili, L. FDA-National Center for Toxicological Research, Division of Nutritional Toxicology, Jefferson, AR 72079

Two days after initiating a folate/methyl deficient diet in weanling F344 rats, the incidence of hepatic apoptotic bodies (without necrosis or inflammation) was significantly increased. Apoptosis was confirmed biochemically by an increase in Ca/Mg-dependent endonuclease activity in nuclear extracts. DNA strand breaks were also detectable in genomic DNA two days after initiating the deficient diet and the activity of the DNA repair-associated enzyme poly(ADP-ribose)polymerase was simultaneously stimulated. The hepatic deoxynucleotide pools were severely depressed after 5 days of deficiency and were associated with major decreases in the nucleotides NAD, ATP and GTP. These changes in nucleotide pools are consistent with an acute energy crisis and an arrest or delay in DNA synthesis and cell cycle progression. These indications of acute biochemical and molecular stress were followed by a transient recovery toward control levels after 5-7 days on the deficient diet. However, after 3-9 weeks of folate/methyl deficiency, significant increases were maintained in hepatic DNA strand break accumulation, DNA repair activity, nucleotide imbalance, apoptotic cell death and regenerative proliferation.

3231

**Molecular pathogenesis of basal cell carcinoma: p53 mutations and instability of chromosome 9.**

D'Errico M<sup>1</sup>, Scio M<sup>1</sup>, Baliva G<sup>1</sup>, Corona R<sup>1</sup>, Signoretti S<sup>1</sup>, Dogliotti E<sup>2</sup>.

<sup>1</sup> Istituto Dermopatico dell'Immacolata (IDI - IRCCS), 00167 Rome, Italy. <sup>2</sup> Istituto Superiore di Sanità, 00161 Rome, Italy.

Basal cell carcinoma (BCC) is the most common skin cancer and the most common malignancy in humans. Its occurrence is clearly correlated with exposure to sunlight. To investigate whether a prolonged exposure to UV light is responsible for the accumulation of mutations in genes critical for tumor development, we analyzed genomic DNA from BCC biopsies for the presence of p53 mutations. Additionally, loss of heterozygosity of chromosome 9q has been found in sporadic and hereditary BCCs. To further define the genetic events occurring during the tumorigenesis, alterations in microsatellite sequences and allele loss in chromosome 9 were also examined. Preliminary results obtained for 12 BCCs show that 3 tumors (25%) present heterozygous mutations in exons 5 to 8 of the p53 gene. All mutations were targeted at dipyrimidine sites and both transitions (C→T) and transversions (C→A and T→G) were identified. Interestingly, one sample showed multiple mutations likely localized on separate alleles. 4/5 mutations were targeted at mutation hotspots specific for skin cancers. The type and distribution of mutations detected in this study strongly support the hypothesis that p53 mutations are caused by exposure to UV light. 3/3 BCCs examined for genetic alterations of chromosome 9 showed major allelic rearrangements of more than one 2-base pair repeat by using a microsatellite marker that maps at 9p22-21, suggesting that microsatellite instability is also associated with BCC.

**#3768** Wednesday, April 24, 1996, 8:00–12:00, Poster Section 12  
Deletion mapping of benign, borderline, and neoplastic sporadic ovarian tumors. Muller C.Y., Vu T., Sharma D.S., Miller D.S., Mathis J.M., and Orth K. *Univ. Texas Southwestern Med. Ctr., Dallas, TX 75235.*

Heterozygosity studies comparing allelic alterations in DNA samples from tumor and normal tissue using polymorphic microsatellite (CA)<sub>n</sub> repeat markers at the p53, the BRCA1, and the BRCA2 gene loci were performed with sporadic ovarian tumors. Matched normal and tumor DNA of 94 ovarian cancer patients (including 4 benign, 30 borderline, 47 malignant epithelial, 6 sex cord, and 7 germ cell cases) was isolated from paraffin embedded tissue by proteinase K digestion and phenol-chloroform extraction. The incidence of allelic loss in tumor DNA was screened using markers linked to the p53 gene on 17p13, the BRCA1 gene on 17q21, the BRCA2 gene on 13q12–13, and control markers on chromosomes 5 and 6. PCR analysis showed LOH at the p53 locus in 4% of informative borderline tumors, 44% of informative malignant epithelial tumors, 17% of informative germ cell tumors. LOH was observed at the BRCA1 locus only in the informative malignant epithelial tumors (54%) and germ cell tumors (33%). Only 2 of 47 malignant epithelial cases (4%) showed alterations in allelic markers demonstrating the replication error (RER+) phenotype described in HNPCC. No LOH was observed in benign tumors at any loci screened. These results indicate that LOH on chromosome 17 is rare in benign and borderline ovarian tumors compared to malignant tumors. LOH at the BRCA2 locus was low in malignant tumors and was not detected in benign or borderline tumors. Thus, allelic loss at the BRCA2 locus is not a significant event in sporadic ovarian cancer.

**#3769** Tuesday, April 23, 1996, 1:00–5:00, Poster Section 5  
Carcinogen and rat strain specificity of an allelic imbalance on chromosome 1 in rat mammary carcinomas. Haag, J.D., Hsu, L-C., and Gould, M.N. *University of Wisconsin, Department of Human Oncology, Madison, WI 53792.*

In order to better understand the genetic components controlling individual rat strain susceptibilities to mammary carcinogenesis, we induced mammary carcinomas in (WFXCop)F1 rats using either DMBA or radiation. Tumors were screened for allelic imbalances using 64 polymorphic microsatellite markers spanning the genome. While random allelic imbalances were observed in both the radiation- and DMBA-induced tumors, a non-random loss of heterozygosity (LOH) was also observed on chromosome 1 in the DMBA-induced tumors. This region spans approximately 40 cM from microsatellite markers R511 to R272 with a maximum frequency of LOH of 54% (6/11 tumors) occurring at the R27 locus. We then screened 3 other subsets of DMBA- and radiation induced mammary tumors for allelic imbalance on Chromosome 1 and found this imbalance only in the DMBA-induced tumors from the (F344xCop)F1. Thus far, only DMBA-induced F1 mammary tumors with a Cop parental strain have shown the chromosome 1 imbalance; however, the imbalance does not favor the Cop parental allele. These results suggest that: 1) rat mammary tumorigenesis occurs via different molecular mechanisms for the two carcinogens, DMBA and radiation, and 2) the genetic background of the F1 rat appears to influence the types of molecular lesions identified in the mammary tumors.

**#3770** Monday, April 22, 1996, 1:00–5:00, Poster Section 14  
No microsatellite instability or consistent loss of heterozygosity in B precursor cell acute lymphoblastic leukemias of children. Hua-Fang Yi and Geoffrey R. Kitchingman. *Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, TN 38101.*

We used 51 microsatellite markers to study all of the chromosome arms from at least 20 patients with childhood B precursor cell acute lymphoblastic leukemia. DNA from diagnosis and remission were studied, as was relapse DNA when it was available. We have found that only the short arm of chromosome 12 is consistently involved in loss of heterozygosity (LOH). Sporadic LOH was found in other chromosomes, but no other microsatellite marker was reduced to homogeneity in more than 10% of the cases studied. This contrasts with what has been found in solid tumors. We are currently expanding the number of markers used genome wide, and we are saturating 12p to define the smallest region of loss. We found no evidence for microsatellite instability. To partially confirm this, six exons of the MSH2 gene were examined for mutations by PCR and SSCP; no alterations were found. Thus, despite the presence of multiple chromosomal abnormalities in the majority of acute leukemias, LOH is infrequent and there are no changes in the microsatellite repeats.

**#3771** Sunday, April 21, 1996, 1:00–5:00, Poster Section 2  
Frequent loss of heterozygosity on chromosome 16q in "in situ" breast cancer. Chen, T.<sup>1</sup>, Paladugu, A.<sup>1</sup>, Sahin, A.<sup>2</sup>, and Aldaz, C.M.<sup>1</sup>. <sup>1</sup>Dept. of Carcinogenesis, UT MD Anderson Cancer Center, Science Park—Research Division, Smithville, TX 78957; <sup>2</sup>Dept. of Pathology, UT MD Anderson Cancer Center, Houston, TX 77030

In our recent study of comparative allelotyping of *in situ* and invasive breast cancer lesions from paraffin embedded samples we determined that allelic losses affecting chromosome arms 16q, 17p, 17q and chromosome 7 appear to be early abnormalities since they were observed in a significant number of ductal carcinoma *in situ* (DCIS) lesions. In this study we performed a high resolution allelotyping of chromosome 16 in DCIS in order to further define the minimal target region for loss of heterozygosity (LOH). We observed a very high frequency (9 of 12 tumors) of LOH affecting the long

arm of this autosome. By overlapping LOH the most commonly affected marker was D16S398 at q22.1 chromosome band. Interestingly the E-cadherin gene (CDH1), a calcium dependent cell adhesion molecule, maps to this same chromosome band. The most common mechanisms for 16q allelic losses in DCIS appear to be mitotic recombination or terminal deletion. In order to better understand the chromosomal events leading to LOH we are analyzing adjacent tissue sections by means of chromosome 16 fluorescence *in situ* hybridization. These studies will assist in the elucidation of the mechanisms and extent of chromosome 16 involvement at preinvasive stages of breast carcinogenesis. (U.S. Army Grant DAMD17-94-J-4078)

**#3772** Wednesday, April 24, 1996, 8:00–12:00, Poster Section 12  
Involvement of a locus which escapes X chromosome inactivation in ovarian tumors of low malignant potential. Dubreau, L., Cheng, P., Kim, T.M., Velicescu, M., Wan, M., Zheng, J., Felix, J.C., Luo, P. *Univ. of Southern California/Norris Comprehensive Cancer Center, Los Angeles, CA 90033.*

Ovarian tumors of low malignant potential (LMP) are a subtype of ovarian epithelial tumors with reduced invasive and metastatic abilities. They are regarded as intermediate between benign (cystadenomas) and malignant (carcinomas) ovarian neoplasms. Allelotyping analyses of 24 LMP tumors showed that losses of heterozygosity in somatic chromosomes are rare in these tumors. Such losses were present in only 6 of the 24 tumors and involved a single somatic chromosome in five cases. In contrast, 8 of 16 (50%) LMP tumors informative for the androgen receptor locus in chromosome Xq showed losses of heterozygosity involving this locus. The losses were interstitial in 6 of the 8 cases. Examination of the methylation status of the retained androgen receptor alleles showed that the losses always affected the inactive copy of the X chromosome. By comparison, allelic losses on chromosome Xq were not seen in 15 low grade ovarian carcinomas. We conclude that a locus which escapes X chromosome inactivation in chromosome Xq is important for LMP tumor development. The results also suggest that low grade ovarian carcinomas do not usually evolve from pre-existing LMP tumors.

**#3773** Tuesday, April 23, 1996, 8:00–12:00, Poster Section 6  
High resolution allelotyping in hepatocellular carcinoma (HCC). Boige V.<sup>1,3</sup>, Laurent-Puig P.<sup>1,3</sup>, Schmitz A<sup>7</sup>, Flejou JF<sup>2</sup>, Bedossa P<sup>3</sup>, Bioulac-Sage P<sup>4</sup>, Monges G<sup>5</sup>, Capron F<sup>6</sup>, Olschewski S<sup>1</sup>, Thomas G<sup>1</sup>. <sup>1</sup>Institut Curie, <sup>2</sup>Hôpital Beaujon, <sup>3</sup>Bicêtre, <sup>4</sup>Béclère, <sup>5</sup>CHU Bordeaux, <sup>6</sup>Centre Paoli-Calmettes, Marseille; <sup>7</sup>CEA Fontenay-aux-Roses.

HCC is one of the most common malignancies in the world. Despite this, the molecular events involved in the development of this neoplasm are not well understood. In order to achieve a broader perspective on the prevalence of allelic deletions in HCC and to analyze the relationship between these alterations, we studied all non acrocentric chromosome arms in a group of 48 HCC. We used for this purpose 275 highly polymorphic microsatellite genetic markers with a mean heterozygosity of 0.75. In total, 1853 of the 1872 chromosome arms (99%) studied were informative for at least one microsatellite loci. Nine chromosome arms were deleted in more than 30% of the tumors (1p, 1q, 4q, 6q, 8p, 9p, 16p, 16q, 17p). The most frequent chromosome arm deletion was observed for 8p (60.4%). The mean of the Fractional Allelic Loss was 0.21. We demonstrated a significant association between LOH occurring on the short and long arms of chromosomes 1 and 16 and between chromosomes 16p and 4q. Furthermore, the high resolution of the allelotyping allowed us to point out some of the smallest common region of deletion among the most frequent arms lost.

**#3774** Tuesday, April 23, 1996, 1:00–5:00, Poster Section 5  
Allelic loss on chromosome 8p12-21 in BRCA1 mutation positive familial breast/ovarian cancer. RA Weiss, E DiFranco, S Aznavoorian, WM Linehan, C Yocke, R Chuquai, MJ Merino, LA Liotta, Z Zhuang, JP Struwing<sup>1</sup>, and MR Emmert-Buck, *Lab of Path, NCI, and NCHGR, Bethesda, MD 20892.*

Frequent loss of heterozygosity (LOH) at specific genetic loci in DNA of tumor cells suggests the presence of a tumor suppressor gene within the deleted region. LOH on the short arm of chromosome 8 has been identified in several carcinomas including colorectal, bladder, hepatocellular, prostatic, and sporadic breast cancer. We studied LOH on 8p in tumors of 21 BRCA1 mutation positive familial breast/ovarian cancer patients. DNA was extracted from microdissected normal and invasive tumor cells obtained from formalin-fixed, paraffin-embedded tissue sections, and analyzed with 7 polymorphic DNA markers on 8p, 1 marker on 8q, and 1 marker on 17q at the BRCA1 gene. Extracted DNA was amplified by the polymerase chain reaction (PCR) and studied for LOH by denaturing gel electrophoresis and autoradiography. LOH was identified with at least 1 marker on 8p in 71% (12/17) of informative cases. The highest rate of deletion was 89% (8 of 9 informative cases) at marker D8S137 on 8p12-21. LOH was not observed on 8q. Seven of 8 (88%) informative cases showed deletion of 17q at BRCA1. Both breast and ovarian cancers demonstrated high rates of 8p LOH. These results suggest that a tumor suppressor gene located at 8p12-21 may be important in the development of BRCA1 mutation positive familial breast/ovarian cancer. Additionally, 8p LOH may represent a convergence of genetic alterations between sporadic and familial breast cancer.

## MOLECULAR BIOLOGY/BIOCHEMISTRY

We have analyzed 150 frozen breast tumors using the TRAP assay to determine the level of activity and processivity of telomerase in these specimens. 91 of 150 tumors (61%) were positive for telomerase activity. Correlative analyses indicated that there were statistically significant correlations between telomerase activity and S-phase fraction (SPF,  $r = 0.23$ ,  $p = 0.004$ ), and EGFR ( $r = 0.20$ ,  $p = 0.013$ ). There was also weak correlation between telomerase activity and Cathepsin D levels ( $p = 0.066$ ), but no statistically significant correlation of telomerase with ploidy, estrogen receptor (ER), and progesterone receptor (PGR) status. Correlative analyses of the processivity of telomerase with each prognostic marker showed statistically significant correlations with SPF ( $p = 0.008$ ) and EGFR ( $p = 0.035$ ), probably due to the strong correlation between processivity and telomerase activity (corr. coef. = 0.75,  $p = 0.0001$ ). We have further analyzed retrospectively another 400 node-positive stage I breast tumors. Result indicated that 323 of 400 (81%) breast cancer samples were positive for telomerase activity. There were statistically significant correlations between telomerase activity and SPF ( $r = 0.26$ ,  $p = 0.0001$ ), ploidy ( $r = 0.17$ ,  $p = 0.0007$ ), and age ( $r = -0.12$ ,  $p = 0.02$ ). Weak or no correlations were observed between telomerase activity and the number of positive nodes, tumor size, ER, and PGR. Processivity was again very strongly correlated to telomerase activity ( $r = 0.69$ ,  $p = 0.0001$ ), but correlated only with ploidy ( $r = 0.15$ ,  $p = 0.009$ ) and SPF ( $r = 0.14$ ,  $p = 0.01$ ). Conclusion: Telomerase is a novel marker that may serve as an important prognostic indicator in human breast cancer.

**#3853** Tuesday, April 23, 1996, 8:00–12:00, Poster Section 5  
**Cytokine regulation of telomerase activity in bone marrow primitive progenitors.** Yui, J., Dragowska, V., Thornbury, G., Lansdorp, P. *Terry Fox Lab, Vancouver, BC, Canada.*

Telomerase is a ribonucleoprotein polymerase which synthesizes telomeric repeats onto the 3' ends of eukaryotic chromosomes. Activation of telomerase prevents telomeric shortening and may confer upon cells an extended lifespan as found in germ cells, immortalized cell lines and tumor tissues. As normal hematopoietic cells express low levels of telomerase, we are interested in examining cytokines that may upregulate functional telomerase activity in stem cell candidates (SCC) so as to potentially enhance their proliferative potential. Human SCC with the CD34<sup>+</sup> CD45RA<sup>lo</sup>CD71<sup>lo</sup> phenotype were purified from adult bone marrow and cultured for four days in stem cell factor (SCF), IL-3 and flt3 ligand (flt3-L). Functional telomerase was detected in SCC on day 0 using the telomeric repeat amplification protocol (TRAP). After 4 days in culture, activity was detected in total cell extracts from SCF + IL-3 and SCF + IL-3 + flt3-L cultures, but not from cells cultured in SCF or SCF + flt3-L. Within the CD34<sup>+</sup> fraction, cells expressing low levels of CD45RA and CD71 had undetectable activity whereas considerable activity was found in the CD34<sup>+</sup>CD71<sup>+</sup> fraction. In addition, cells that have undergone proliferation as determined by loss of PKH26 staining showed positive telomerase activity whereas PKH26<sup>bright</sup> cells lack telomerase activity. These results suggest that in adult bone marrow, telomerase activity is associated with proliferating and differentiating hematopoietic progenitors but may not be expressed in quiescent stem cells.

**#3854** Tuesday, April 23, 1996, 8:00–12:00, Poster Section 5  
**Inhibitory effects of telomere-mimic phosphorothioate oligonucleotides on various human tumor cells in vitro.** Ohnuma, T., Li, F-L., Zon, J., Zon, G., Pogo, B. and Holland, J.F. *Mount Sinai Medical Center, New York, NY 10029 and Lynx, Hayward, CA.*

Telomerase, a ribonucleoprotein that synthesizes telomeric DNA onto chromosomal ends, is known to be expressed in malignant tumor cells. We tested the cell growth inhibitory effects of telomere-mimic oligomers, 5'-d(TTAGGG)<sub>n</sub>-3' where  $n = 1, 2, 3$  or 4 in the following 9 human tumor cell lines: 2780 ovarian carcinoma, Hep-2 squamous cell carcinoma, VAMT-1 mesothelioma, DND-1A melanoma, MOLT-3 ALL, Jurkat lymphoma, Daudi Burkitt lymphoma, JAR choriocarcinoma and SK-LMS sarcoma. As controls, 2 scrambled 24-mers were tested. Among the compounds tested, 6-mer was not active in any of the cell lines studied. Increases in the length of oligonucleotides from 12- to 18- and 24-mer resulted in increased cell growth inhibitory activity in sensitive cell lines. Cells in suspension cultures, MOLT-3 ALL and Daudi Burkitt lymphoma were generally more sensitive than the monolayers (24-mer ID<sub>50</sub> = ~3  $\mu$ M). While inhibitory effects of authentic 24-mer oligomer were more pronounced than the scrambled oligomers, both of the scrambled 24-mers also showed some degree of inhibitory activity. Except for modest activity of the 24-mer in 2 cell lines, DNA-1A and 2780, none of the compounds tested were active against solid tumor cell lines. These data indicate that further study of the telomere-mimic 24-mer is warranted as a candidate compound for the treatment of leukemia/lymphoma.

**#3855** Sunday, April 21, 1996, 04:25–04:40, Room 33  
**Telomerase in human breast cancer.** Bednarek, A.<sup>1</sup>, Sahin, A.<sup>2</sup>, and Aldaz, C.M.<sup>1</sup>  
<sup>1</sup>*Dept. of Carcinogenesis, Univ. of Texas M.D. Anderson Cancer Center, Science Park-Research Division, Smithville, TX 78957; <sup>2</sup>Dept. of Pathology, Univ. of Texas M.D. Anderson Cancer Center, Houston, TX 77030*

Telomerase is a ribonucleoprotein enzyme which contains RNA template complementary to telomeric repeats (TTAGGG) that permits the *de novo* synthesis of telomeric DNA. It has been postulated that malignant tumors must express telomerase activity to maintain their immortality. Unlike tumors, in most human adult tissues, telomerase

activity is not detected. To investigate the role of telomerase in breast cancer and to analyze its putative association with parameters of diagnostic-prognostic significance we assayed the activity levels of this enzyme in a set of 94 primary breast cancers. These studies were performed by means of the very sensitive telomeric repeat amplification protocol. To compare the level of telomerase activity in the different tumor samples we used a semiquantitative analysis based on the use of an internal standard. Telomerase activity was detected in 91 (96.8%) tumors, only in 3 (3.2%) activity was undetected. Telomerase activity was detected in 3 of 3 ductal carcinomas *in situ* which constitute a preinvasive stage. Most tumors negative for lymph node metastasis were also positive for telomerase activity. On the other hand 10 of 10 examined adjacent normal tissues showed no telomerase activity. The data obtained showed no apparent association between telomerase activity levels and estrogen receptor status, s-phase, ploidy or lymph node metastasis status. These data indicate that telomerase activation could constitute a relative early event in breast carcinogenesis. Therefore telomerase may be a useful target for therapeutic intervention at early breast cancer stages. (US Army Grant DAMD17-94-J-4078)

**#3856** Tuesday, April 23, 1996, 8:00–12:00, Poster Section 5  
**Telomerase activity distinguishes different subtypes of epithelial ovarian tumors.** Wan, M., Duggan, B.D., Li, W., Felix, J.C., Zhao, Y., Dubeau, L. *Univ. of Southern California/Norris Comprehensive Cancer Center, Los Angeles, CA 90033.*

Telomerase is a RNA-dependent DNA polymerase that directs the synthesis of telomeric DNA repeats onto the ends of eukaryotic chromosomes. It compensates for the telomeric losses that occur with each round of DNA replication. Telomerase is repressed in most normal cells after fetal life but its reactivation is thought to be essential for continuous cell division such as occurs in malignant tumor cells. We examined telomerase activity in 53 ovarian epithelial tumors, subdivided into cystadenomas (benign), tumors of low malignant potential (LMP), and carcinomas (malignant) in order to further examine the role of this enzyme in ovarian tumor development. Telomerase activity was detected in 20 (100%) of 20 carcinomas and in 17 (100%) of 17 LMPs. In contrast, telomerase expression was present on only 4 (25%) of 16 cystadenomas. Most of the positive cystadenomas belonged to a rare subtype called papillary. These results suggest that telomerase may be a useful marker for the detection of ovarian LMP tumors and carcinomas in clinical samples such as ascitic fluids. The data also suggest that the underlying mechanisms leading to ovarian papillary cystadenoma development may be different than for typical cystadenomas and that the papillary tumors may best be reclassified as LMP tumors.

**#3857** Sunday, April 21, 1996, 03:25–03:40, Room 33  
**The effect of 7-deaza-2'-deoxynucleoside triphosphates on telomeres and telomerase.** Fletcher, T. M. & Chen, S. F. *Cancer Therapy and Research Center Institute for Drug Development, San Antonio, TX 78245.*

Telomeres play an important role in chromosome organization and stability. Human telomerase is a terminal transferase that adds TTAGGG units onto the telomere end. In general, telomerase activity is not detected in normal somatic cells but is present in immortalized cells. Consequently, telomerase might be a selective target for cancer chemotherapy. Using an *in vitro* telomerase assay, we have found that 7-Deaza-2'-Deoxyguanosine-5'-Triphosphate (7-Deaza-dGTP) and 7-Deaza-2'-Deoxyadenosine-5'-Triphosphate (7-Deaza-dATP) were potent telomerase inhibitors. The concentration of inhibitors in which 50% of the telomerase activity was inhibited (IC<sub>50</sub> values) were 6–8  $\mu$ M for both nucleotides. Additional studies show that 7-Deaza-dGTP and 7-Deaza-dATP are also incorporated into telomeric DNA by telomerase. However, incorporation of 7-Deaza-dGTP results in a telomeric ladder that is prematurely shortened. It is possible that the telomerase translocation step is inhibited because 7-Deaza-dGTP lacks the 7 nitrogen which is essential for the formation of G-quartets or hairpins. This may be a novel approach in the design of new telomerase inhibitors. (Supported in part by Sanofi Winthrop Research and in part by a NIH-sponsored NCDDG grant.)

**#3858** Tuesday, April 23, 1996, 8:00–12:00, Poster Section 5  
**Telomerase activity in hematopoietic cells.** Leteurtre F (1), Gluckman E (2), Carosella E (1). *Service de Recherches en Hématologie, DRM, CEA (1) & Bone marrow Transplantation Unit (2); Hôpital Saint-Louis, 75745 Paris cedex 10, France.*

Telomeric DNA protects chromosome ends from recombination events and its length may serve as a mitotic clock that signals cell senescence and exit from cell cycle. Telomerase synthesizes *de novo* telomeric DNA and, authorizes unlimited cell proliferation. An hematopoietic dogma proposes an unlimited self renewal of hematopoietic stem cell (HSC). Using a recently described PCR-based measure of telomerase activity, we looked for the enzyme activity in hematopoietic cells. In agreement with the dogma, telomerase activity was found in mononuclear fractions containing HSC: adult bone marrow and cord blood cells. However, normal leukocytes also expressed the enzyme activity. Thus, telomerase activity is not a marker of HSC. Large variation in the intensity of telomerase activity was observed in normal leukocytes from healthy volunteers. This observation and that of telomerase activity in the leg bud of a 30 day fetus and in its trophoblast suggest that telomerase might be activated in some proliferating tissues. Therefore, telomerase activity is currently under investigation in leukocytes from patients with inflammatory response, in advanced aplasia and in hematopoietic

## GY/BIOCHEMISTRY

p21 monoclonal antibody. The protein expression peaked at day 5 after infection. A high level of expression of exogenous p21 was achieved in the H1299 cells and H460 cells infected by Ad5CMV-p21 (200 MOI), but expression in H322 cells was lower. Ad5CMV-poly A infected cells and mock-infected cells failed to show p21 staining. The growth of the Ad5CMV-p21-infected cells was greatly suppressed as evidenced by a decrease of both [<sup>3</sup>H]thymidine incorporation and cell counts. Ad5CMV-p53 was compared with Ad5CMV-p21. The MOIs for 50% inhibition (IC<sub>50</sub>) by [<sup>3</sup>H]thymidine incorporation assay for H1299, H322 and H460 were 64.8, 49.7 and 60.5 for Ad5CMV-p21, and 19.2, 26.9 and 78.7 for Ad5CMV-Ad5CMV-p53.

These data suggest that transduction of wildtype p21 can mediate inhibition of non-small cell lung cancer growth.

### #4049 Wednesday, April 24, 1996, 8:00-12:00, Poster Section 12

**The role of p16, p15, and p18 in ovarian cancer.** Emilie Marioli<sup>1</sup>, Gerardo Arroyo<sup>1</sup>, Jeffrey Struewing<sup>2</sup>, Robert Taylor<sup>3</sup>, Joseph Geradts<sup>4</sup>, Michael Birrer<sup>1</sup>. <sup>1</sup>BPRB, DCS, NCI, Rockville MD 20850; <sup>2</sup>DCEG, NCI, Bethesda MD 20892; <sup>3</sup>Walter Reed Army Medical Center, Washington DC 20307; <sup>4</sup>University of North Carolina, Chapel Hill NC 27599.

We characterized the p15, p16, and p18 genes in 30 matched tumor and normal samples from patients with sporadic ovarian cancer (SOC), and the p16 gene in 8 patients with familial ovarian cancer (FOC). PCR amplification and agarose gel electrophoresis demonstrated the presence of all exons of these genes in the tumor samples. 1/30 (3%) of the SOC, and 1/8 (12%) of the FOC specimens had an abnormal SSCP pattern in the p16 analysis. Cloning and sequencing revealed an A->C transversion at the first base of codon 140, resulting in the substitution of the aminoacid thr for ala. Normal DNA from these two patients showed the same base substitution, suggesting the presence of polymorphism. 1/30 (3%) of the SOC specimens revealed an abnormal pattern in the SSCP analysis of the p15 gene in normal and tumor DNA. SSCP analysis of p18 revealed no abnormality in any of the samples analyzed. Because point mutations are not the only mechanism for inactivation of p16, protein expression was analyzed by immunohistochemistry, in the SOC cases. 1/30 (3%) of the SOC specimens revealed no staining for p16 in the tumor, and 1/30 (3%) of the SOC specimens (a tumor with biphenotypic histology) stained only in the squamous area but not in the region with adeno differentiation. The 2 samples with the p16 substitution had normal protein expression. We conclude that p16, p15 and p18 genes appear to have a minor role in ovarian cancer.

### #4050 Tuesday, April 23, 1996, 1:00-5:00, Poster Section 7

**Von Hippel-Lindau (VHL) gene expression in renal cell carcinoma.** Vasavada, S.P., Ye, Y., Stackhouse, T., Kuzmin, I., Lerman, M., Zbar, B., Williams, B.R.G. Cleveland Clinic Foundation, Cleveland, OH 44195 and the National Cancer Institute, Frederick, MD 21702

The Von Hippel-Lindau (VHL) tumor suppressor gene has a critical role in the pathogenesis of renal cell carcinoma. We have previously demonstrated elevated levels of VHL mRNA in the proximal convoluted tubules of the kidney, the site of origin of renal cell carcinoma. We have now examined the cellular expression pattern for VHL in several renal cell carcinoma cell lines to further localize the protein and help determine its ultimate function. Four characterized renal cell carcinoma cell lines were examined with polyclonal antibodies to N and C-terminal ends of the VHL gene with immunofluorescent cytochemistry. Subsequent nuclear stains were performed with anti-splicesome antibody to determine nuclear colocalization. Cells were also examined at differing degrees of confluence on culture plates. Both immunofluorescence and confocal microscopy demonstrated a fine speckled nuclear staining pattern. Subconfluent cells exhibited moderate cytoplasmic staining and subsequent colocalization with antislicesome antibody revealed a pattern distinct from that of nuclear chromatin. Confluent cells exhibit almost exclusive nuclear staining. These findings are consistent with the transcriptional role postulated for VHL and suggest that nuclear translocation of the protein may occur, subject to cell density.

### #4051 Sunday, April 21, 1996, 8:00-12:00, Room 20

**Comprehensive analysis of the p16<sup>INK4A</sup> gene and transcripts in breast cancer.** Bremer, A.J.<sup>1</sup>, Paladugu, A.<sup>1</sup>, Dreyling, M.H.<sup>2</sup>, Olopade, O.I.<sup>2</sup>, Wang, H.<sup>1</sup>, Aldaz, C.M.<sup>1</sup>. University of Texas M.D. Anderson Cancer Center, Science Park<sup>1</sup>, Smithville, Texas 78957; Department of Medicine<sup>2</sup>, University of Chicago, Chicago, Ill, 60637

Homozygous deletion and loss of heterozygosity (LOH) of chromosomal subregion 9p21 are a frequent occurrence in a variety of primary tumor types. We have recently reported a high rate (58%) of LOH of this region in breast cancer as well. The p16<sup>INK4A</sup> tumor suppressor gene has been localized in this region, and thus has been implicated in the genesis or progression of these tumors. However, when mutational analysis by sequencing and SSCP was conducted in these same tumors, we found aberrations to be infrequent, thus contradicting the involvement of this gene as postulated by Knudson's two hit hypothesis. Recent reports have suggested the primary means of p16<sup>INK4A</sup> inactivation may alternatively be through homozygous loss or methylation of CpG islands in the 5' region of this gene. To address this issue, we have performed interphase cytogenetics fluorescence *in situ* hybridization (IC-FISH) with a 250Kb p16<sup>INK4A</sup> cosmid contig as well as methylation sensitive restriction endonuclease Southern analysis in these tumors. Additionally, through reverse transcription coupled PCR we have analyzed the level of expression of the p16<sup>INK4A</sup> transcript and the recently

## MOLECULAR BIOLOGY

reported beta transcript which is encoded from a separate promoter and first exon but with the same exon 2. In initial analyses utilizing this comprehensive approach, we have observed p16 to be affected in greater than 50% of human breast cancer samples analyzed thus far. (U.S. Army Grant DAMD17-94-J-4078)

#  
D  
R  
H  
E  
M  
o  
U  
h  
D  
e  
N

2  
S  
T  
7  
d  
R  
c  
a  
n  
d  
t  
I  
e  
c  
t

I  
I  
J  
C  
R  
C  
V  
S  
I  
C  
T  
C  
I  
F  
I  
T  
C  
I  
F  
I

**#4052 Wednesday, April 24, 1996, 8:00-12:00, Poster Section 12**  
**Introduction of a normal human chromosome 6 into ovarian carcinoma cell lines controls their tumorigenic expression.** Wan, M., Vyas, R., Zheng, J., Dubeau, L. Univ. of Southern California/Norris Comprehensive Cancer Center, Los Angeles, CA 90033.

Structural abnormalities involving chromosome 6 are among the earliest and most consistent chromosomal alterations described in human ovarian carcinomas. We and others previously demonstrated that three distinct regions of chromosome 6 are frequently affected by losses of heterozygosity in primary human ovarian carcinomas. One of these regions involves the middle portion of chromosome 6q, a region also deleted in the ovarian carcinoma cell lines SK-OV3 and HEY. The role of this deletion in malignant transformation was investigated by introducing a normal human chromosome 6 into the SK-OV3 and HEY cell lines using microcell-mediated chromosome transfer techniques. The normal chromosome 6 was stably propagated in some hybrids as shown by fluorescence *in situ* hybridization (FISH). Tumorigenicity and anchorage-independent growth of SK-OV3 and HEY cells were completely suppressed or reduced after such transfer. In contrast, transfer of chromosome 11q in the same cell lines showed no measurable phenotypic changes. These results strongly support the hypothesis that the middle portion of chromosome 6q contains a tumor suppressor gene important for ovarian carcinoma development.

**#4053 Wednesday, April 24, 1996, 8:00-12:00, Poster Section 12**  
**Potential tumor suppressor loci on chromosome 14q in invasive epithelial ovarian cancer.** Bandera, C., Takahashi, H., Rubin, S., and Boyd, J. Department of Obstetrics and Gynecology, University of Pennsylvania, Philadelphia, PA 19104.

Chromosome 14 was screened for possible tumor suppressor loci in invasive epithelial ovarian cancer. DNA samples from 73 ovarian carcinoma specimens and matched normal tissues were evaluated using 12 polymorphic microsatellite markers. Loss of heterozygosity (LOH) was identified at one or more loci in 44 cases (60%). In 18 of these 44 cases (41%), at least 50% of informative sites exhibited LOH. In 8 of these 44 cases (18%) all informative sites exhibited allelic loss suggesting loss of the entire long arm of chromosome 14. An increased rate of LOH (34%-37%) was noted at three loci on 14q including D14S70, D14S290 and D14S267 mapping to 14q12-13, 14q21-23 and 14q32.1-32.2. LOH on chromosome 14 was seen at all stages of disease; however 4 borderline ovarian tumors also screened showed no LOH on chromosome 14. These data show that LOH on chromosome 14 is common in ovarian cancer, and suggest that tumor suppressor genes contributing to the disease process may exist in the regions 14q12-13, 14q21-23 and 14q32.1-32.2.

**#4054 Tuesday, April 23, 1996, 1:00-5:00, Poster Section 7**  
**Molecular cloning and TNF- $\alpha$  mediated expression of the human doc-1 oral tumor suppressor gene.** Tsuji, T., McBride, J., Todd, R., Nagata, E., Donoff, R.B., Liao, P.H., Chou, M.Y. and Wong, D.T.W. Harvard School of Dental Medicine, Boston, MA.

We have recently identified and cloned a novel cellular gene doc-1 from normal hamster oral keratinocytes which exhibits tumor suppressor function when expressed in transformed hamster oral keratinocytes (FASEB J. 9, 1362-1370, 1995). Using hamster doc-1 specific primers, the human doc-1 cDNA homologue has been cloned from total RNA isolated from 2 normal human oral keratinocyte cultures (OKB2 and OKF4). Amino acid analysis of the coding region demonstrated complete identity to that of the hamster doc-1 amino acid coding sequence. Subjecting doc-1 sequence for a FASTA search revealed a significant match to a novel mouse gene TU-166, induced in mouse fibroblasts by TNF- $\alpha$ . These data suggested that doc-1 might be a downstream event in the signaling pathway mediating the cytostatic effects of TNF- $\alpha$ . To address this hypothesis, three human oral (SCC 15, 25, 66) and one cervical (A431) cancer cell lines were phenotyped for TNF- $\alpha$  inducibility of doc-1. All four cell lines were found to express TNF- $\alpha$  Type I and II receptor mRNAs by RT-PCR. Incubation of these human cancer cell lines with human recombinant TNF- $\alpha$  at 400U/ml for 4 hours led to the expression of doc-1 mRNA in all four cell lines. These results link TNF- $\alpha$  to the expression of doc-1, implicating doc-1 as a potential downstream mediator in the tumorigenic pathway of TNF- $\alpha$ . We hypothesize that TNF- $\alpha$  induced specific cellular factors which bind to TNF- $\alpha$  responsive elements in the promoter regulatory region of the human doc-1 gene lead to the induction of doc-1 expression. These results allow us to conclude that TNF- $\alpha$  induces doc-1 expression in human oral keratinocytes, linking the signaling pathway of a tumorigenic cytokine to an oral cancer suppressor gene. (Supported by DE-08680, DE-00275, DE-00318.)

expanding tumor margins (44% vs 24%), Crohn's-like lymphoid reaction (67% vs 24%), intratumoral (85% vs 44%) and peritumoral (100% vs 52%) lymphocytic infiltrates. Survival of patients with localized CRC (Dukes B) was similar regardless of tumor RER status. However, the Kaplan Meier survival curve for patients with RER+ CRC and nodal metastases (Dukes C) was significantly improved compared to similarly staged RER- patients. The ability to better identify HNPCC cases based on pathologic and molecular criteria will greatly aid clinical decision-making with respect to treatment and genetic counselling. \*p<0.05 by X<sup>2</sup>

**#1002 Microsatellite instability at 11p15 markers in Wilms tumor is associated with the loss of heterozygosity in the same region.** Perotti, D., Toffolo, E., Mondini, P., Pilotti, S., Luksch, R., Pession, A., Pierotti, M.A., Fossati-Bellani, F., and Radice, P. *Istituto Nazionale Tumori, Milano, Italy, University of Bologna, Italy*

A molecular study performed on 34 sporadic Wilms' tumors revealed microsatellite instability (MSI) at chromosome 11p15 in five cases (15%). The phenomenon occurred at D11S988 and D11S909 loci, and was specific of this particular chromosomal region since no MSI was detected in 25 additional microsatellite markers mapped to 9 different chromosomes. We did not find any correlation between clinical-pathological data and presence of MSI, but comparing loss of heterozygosity (LOH) and MSI results, it appeared that all the cases with MSI presented a concomitant LOH for 11p15 markers. Conversely, MSI occurred in roughly 40% of tumors bearing LOH for the 11p15 region. In our opinion, noteworthy findings are the site specificity of MSI, the involvement of 11p15 band, and the possibility of considering the loss of one allele and the concomitant instability of the remaining one as a "two hits" inactivation mechanism of putative tumor suppressor gene(s). Partially supported by "Associazione Bianca Garavaglia" and "Associazione Italiana per la Ricerca sul Cancro (AIRC)".

**#1003 p53 nuclear accumulation (p53NA), Bcl-2 expression and clinicopathological characteristics of primary colorectal adenocarcinomas (CAC) with microsatellite instability (MSI).** Manne, U., Shibata, D., Tsao, J., Srivastava, S., and Grizzle, W.E. *University of Alabama at Birmingham, Birmingham, AL 35294, University of Southern California, Los Angeles, CA 90033, National Cancer Institute, Bethesda, MD 20892*

Mutations in mismatch repair genes result in MSI in 10–20% of sporadic colorectal cancers. We have examined 103 archival tissue samples of sporadic primary CAC to evaluate MSI and to determine its association with p53NA, Bcl-2 expression and clinicopathological characteristics. The MSI at four loci (Mfd 27, Mfd 41, Mfd 47, Mfd 57) was analyzed by polymerase chain reaction and p53NA and Bcl-2 expression were detected by immunohistochemistry. MSI was detected in 15% tumors and p53NA was observed in 13% of MSI+ tumors as compared to 47% of MSI- tumors (p=0.004). A similar incidence of Bcl-2 positivity was observed in both MSI+ and MSI- tumors (58% versus 56%). The majority of MSI+ tumors arose in proximal to the splenic flexure (80%), however, no significant difference was found in the distribution of MSI+ tumors between the proximal and the distal colorectum (39 and 49 respectively). The incidence of MSI+ tumors were higher among female patients (27% of 33) as compared to tumors from male patients (9% of 70) (p<0.04). Eighteen percent of 51 mucinous tumors and 12% of 52 non-mucinous tumors have demonstrated MSI. These preliminary data suggest that a subset of tumors negative for p53NA that arise in the proximal colon and from female patients are most likely to exhibit MSI.

**#1004 Frequent deletion in the DNA mismatch repair gene hMSH3 in endometrial cancers with microsatellite instability.** Swisher, E., Mutch, D., Kowalski, L., Herzog, T., Rader, J., and Goodfellow, P. *Washington University, St Louis, MO 63110*

The genetic basis for microsatellite instability (MI) or the replication error phenotype (RER+) exhibited by a subset of endometrial cancers has not been identified. The DNA mismatch repair genes important in colorectal tumorigenesis (hMLH1 and hMSH2) are not involved in endometrial tumorigenesis. In an attempt to clarify the origin of MI in sporadic endometrial cancers, 28 RER+ endometrial cancers were screened for mutations of the human DNA mismatch repair gene hMSH3 using single strand conformation variant analysis of all 24 exons. All variants were sequenced. An identical single base deletion resulting in a truncated protein was discovered in 6 tumors (21.4%). This deletion occurred in a run of 8 consecutive A/T base pairs. Because simple repeat sequences are unstable in cells with defective mismatch repair, the mutation may be an effect of RER rather than a cause. Of 5 informative cases with this mutation, none exhibited loss of heterozygosity at intragenic markers as might be expected if the RER phenotype was due to loss of hMSH3 function. Additionally, 3 novel polymorphisms within this gene were identified, and a difference from the published sequence at codon 620 was confirmed in our population. Because of the inherent instability of simple nucleotide repeats in RER+ cancers, it is important to clarify the causality of any mutations identified in candidate DNA mismatch repair genes.

**#1005 Microsatellite instability in intestinal type gastric cancer.** Hamamoto, T., Yokozaki, H., Yasui, W., Semba, S., Yunotani, S., Miyazaki, K., and Tahara, E. *Hiroshima University School of Medicine, Hiroshima, Japan, Saga Medical School, Saga; Japan*

To elucidate the presence of genetic instability in the gastric precancerous lesions, we searched for the microsatellite alterations in 15 cases of intestinal type gastric cancer at 9 microsatellite loci of (CA)n or (A)n repeats. Alterations at one or more loci were observed in 40.0% of cancers and 26.7% of intestinal metaplasias. Altered metaplastic mucosas were microdissected and reexamined to analyze topographically. All the microsatellite alterations in metaplastic mucosas were detected in adjacent mucosa to the respective cancers. Moreover, in one case, an identical and sequential microsatellite alteration was found in the cancer tissue as well as in the adjacent metaplastic mucosa, suggesting sequential development of gastric cancer from intestinal metaplasia. Frequent alteration was found at the microsatellite locus D1S191, indicating this locus might be altered early in the development of intestinal type gastric cancer. No significant association between microsatellite instability and p53 immunoreactivity was observed in the cases examined. These results overall indicate that microsatellite instability may occur as an early event of stomach carcinogenesis of the intestinal type cancer.

**#1006 Characterization of breast tumor DNA by multiplex microsatellite PCR.** Beckmann, A., Zanker, K.S., and Brandt, B. *Institute of Clinical Chemistry and Laboratory Medicine, Muenster, D-48129, Germany, Institute of Immunology, Witten, D-58453, Germany*

Genomic instability detectable by polymorphic microsatellite markers is a common characteristic of human malignancy. In the case of breast tumors several microsatellite marker analyses are published. The articles showed only frequencies of loss of heterozygosity (LOH) or replication error (RER) less than 50%. For a screening of breast tumors with a high probability of LOH or RER and for the need of only small amounts of DNA we developed a multiplex polymerase chain reaction method consisting of several simple sequence repeats that have a heterozygosity over 80% and a frequency of LOH over 20%. In two multiplex PCRs 3 different microsatellites were amplified (D11S907, D6S261, D6S300 and D11S925, D8S272, D11S927). Primers were developed to generate fragments of different length to allow a simultaneous separation by gel electrophoresis on an automated sequencer. We showed that 63% of 70 analyzed breast tumors had a RER in at least one loci and an additional 28% had a LOH. 50% of the tumors showed multiple alterations. The method provides the basis for the detection and characterization of tumor cells in body fluids and tissue.

**#1007 Microsatellite instability as a predictor of second breast cancer.** Cunningham, J.E., Wang, H., Sahin, A., Mastromarino, C.L., Bondy, M.L., and Aldaz, C.M. *Dept. of Carcinogenesis [C.M.A., H.W.J.], UT MD Anderson Cancer Center, Science Park-Res. Div., Smithville, TX 78957, Depts. of Epidemiology [J.E.C., C.L.M., M.L.B.] and Pathology [A.S.], UT MDACC, Houston, TX 77030*

We recently (Aldaz et al., Cancer Res. 55: 3967–81, 1995) found microsatellite instability (MI) in breast cancers (BC's) from patients with bilateral breast primaries, and hypothesized MI may predict risk for a second breast primary. In an ongoing molecular epidemiologic case-control study we are examining the prevalence of MI in tumors from women with bilateral vs unilateral BC. Five loci are being used: DM (CTG), D3S1298 (CA), D8S322 (GAAA), D18S51 (GAAA) and D21S1245 (GAAA). To date, 27 tumors from 17 bilateral BC patients have been analyzed. MI, defined as 3 or more altered loci, was found in 46% (6/13) of first and 36% (5/14) of second BC's. This prevalence is in agreement with our earlier observations of bilateral patients, and significantly higher than our previous findings in unilateral cases. Where both primary tumors were available from the same patient (9 cases) there was 89% concordance in MI. None of the patients appears to be from an HNPCC, BRCA-1 or BRCA-2 kindred. There was no association with nodal status. These preliminary data support our hypothesis. We are continuing to study additional bilateral patients of various histologies, and matched unilateral controls. (Supported by US Army Grant DAMD17-94-J-4078, MDACC PRS, and Estee Lauder Breast Cancer Research Program.)

**#1008 Use of mononucleotide repeat microsatellites to determine the replication error status of colorectal carcinomas.** Hamelin, R., Hoang, J.M., Zhou, X.P., Cottu, P., Salmon, R.J., and Thomas, G. *Institut Curie, Paris, 75005, France*

Microsatellite instability (MI) is the consequence of the inactivation of mismatch repair genes characterizing a replication error phenotype (RER+). MI was first described in colorectal tumors from hereditary non-polyposis colorectal cancer (HNPCC) and sporadic patients. Colorectal tumorigenesis in RER+ and RER- tumors probably follow alternative pathways, and differences in prognosis have been shown between these two types of tumors. The study of the RER status of a tumor may thus be important in the future in order to determine biological prognosis factors and investigate therapeutic strategies. We have already shown that PCR amplification of Bat-26, a quasi-monomorphic mononucleotide repeat microsatellite (MRMS), was able to confirm the RER status of 159 out of 160 colorectal tumors and cell lines. We analyzed other MRMS localized in coding and non-coding sequences. All of them show a higher degree of instability in RER+ tumors than the more commonly used dinucleotide repeats. Some are quasi-monomorphic like Bat-26, while others show polymorphic alleles in normal DNA samples. Moreover, the average size variation between normal alleles and unstable alleles in RER+ tumors is different according to the analyzed MRMS. We compared these properties for all MRMS and showed that Bat-26 was the most useful for establishing the RER status of colorectal tumors.

these genes; we created a BAC and P1 map of the region defined by YAC 928F9. The breakpoints cluster into two distinct regions within the contig suggesting a very large gene or 2 or more different genes may be involved. We have tested 32 ESTs (between D8S279 and D8S286) for position in the contig and ruled them out as candidate genes. In addition, we have identified the chromosomal partner on the rearranged chromosome in MDA-MB-361 to be chromosome 17 by chromosome painting. FISH with cosmids mapping to chromosome 17 on metaphase chromosomes suggests that the breakpoint is distal to ERBB2 on chromosome 17q. Our molecular analysis of the marker chromosome in MDA-361 also suggest the rearranged chromosome is highly complex containing duplications and deletions of some chromosome 8 and chromosome 17 probes used in this study. Further fine mapping of breakpoints and candidate gene analysis may allow cloning of an oncogene commonly rearranged or amplified in breast cancer.

**#871 Genetic Characterization and Expression of Uridine Phosphorylase.** Deliang Gao, Meng-Ping Liu, R. E. Handschumacher and G. Pizzorno. Dept. of Internal Medicine, Yale Univ School of Medicine, New Haven, CT.

Inhibition of uridine phosphorylase (UPase) by benzylacyclouridine (BAU) results in the elevation of plasma uridine concentration leading, in animal models, to the reduction of the toxic effects of 5-fluorouracil (5-FU). Currently the clinical value of this observation is being evaluated in a trial of 5-FU followed by oral BAU administration. More interestingly, in human breast cancer tissues it was found an increased UPase activity combined with a lower sensitivity to BAU inhibition. Total RNA from human breast cancer tissue specimens was screened, using normal human UPase cDNA as a probe, and it was found that an extra band appeared at lower stringency. This band was not seen in normal tissues, colon tumors and human cell lines in which UPase is BAU-sensitive. These data might suggest that a UPase-similar, but with different enzyme kinetic properties, gene or UPase mutant being present in human breast tumors. Presently, the partial sensitivity to BAU is explored for a possible clinical therapeutic utilization. In order to have a deeper insight in its biological functions, expression regulation, and possibly its origin in evolution, we have characterized the mouse UPase gene. This gene consists of 9 exons ranging in length from 60 to 211 bp, separated by 8 introns with typical donor and acceptor sites. The regulatory elements in its promoter region and its chromosomal localization are now under investigation.

**#872 Characterization of transcripts from a commonly deleted area of chromosome 16 (q23.3-q24.1) in human breast cancer.** Bednarek, A.K. and Aldaz, C.M. Department of Carcinogenesis, University of Texas M.D. Anderson Cancer Center, Science Park-Research Division, Smithville, TX 78957.

We have previously determined that the region q23.3-q24.1 from the long arm of chromosome 16 is frequently affected by allelic losses in human breast cancer. LOH in the region spanning from marker D16S515 to D16S504, was found in a majority (77%) of *in situ* breast carcinomas (Cancer Research 56: 5605, 1996). This suggests that a candidate tumor suppressor gene or genes at this location may play an important role in breast carcinogenesis. To identify areas of hemi- or homozygous loss, we performed a high resolution allelotyping of the chromosome 16q area in a panel of 23 human breast cancer lines. In agreement with our previous findings, most breast cancer lines showed evidence of hemizygosity affecting all or almost all the chromosome 16q arm. One breast cancer line showed a homozygous loss affecting markers from this area, indicating that a likely target gene for inactivation may reside within this region. In order to identify candidate genes, we built a contig of YAC and BAC clones spanning the target region. Using a high density STS map we determined that the deletion is approximately 300 kb in size. Using BAC clones from this region, as selector DNA, we have isolated numerous cDNA clones from a human breast cDNA library. Partial sequencing and sequence comparison with GenBank databases indicate that these transcripts represent novel genes. We are currently characterizing the cDNA clones mapping to this region. (Supported by US Army Grant DAMD 17-94-J-4078)

**#873 Localization Of 1p36 Breakpoints In Hematological Malignancy.** Varga, Andrea E., Webb, Graham C., Hutchinson, Rhonda, Weith, Andreas; Dobrovic, Alexander. Departments of Haematology-Oncology, Medicine and Obstetrics & Gynaecology, The Queen Elizabeth Hospital, Woodville, SA 5011, Australia; Research Institute of Molecular Pathology, Vienna, A-1030, Austria.

We used P1 artificial chromosomes (PACs) with inserts at intervals along chromosome band 1p36 to study patients with diverse chromosome rearrangements involving 1p36 as a first step to identifying new genes involved in leukaemia pathogenesis. Three patients with 1p36 abnormalities were studied: an AML patient with inv ins(12;1)(p12;p21p36), an MDS patient with t(1;3)(p36;q21) and a NHL patient with dup ins(1)(p36;q21 q44). The breakpoints were mapped relative to the PAC inserts by fluorescence *in situ* hybridisation (FISH). All three breakpoints mapped to the distal portion of 1p36. As at least one gene in this region is imprinted, it may be possible that the rearrangements might act as the second hit to disrupt an imprinted tumour suppressor gene.

**#874 An improved PEP-PCR variant for multiple microsatellite and sequence analysis of DNA from microdissected single or few tumor cells.** Dietmaier, W., Hartmann, A., Heinmoller, E., Kerner, T., Wallinger, S., Jauch, K.W., and Rüschhoff, J. Institute of Pathology, Department of Surgery, University of Regensburg, D-93042 Regensburg, F.R.G.

Whole genome amplification is a very useful tool for analysis of single or few cells, allowing multiple rather than single DNA analyses. We have tested the efficiency of *Primer-Extension-Preamplification-PCR* (PEP; Zhang et al., Proc. Natl. Acad. Sci., Vol. 89: 5847-5851, 1992), *Degenerate Oligonucleotide Primer-PCR* (DOP) and our new PEP-variant (I-PEP, improved PEP) on FACS sorted and microdissected tumor cells by subsequent single-round PCR. The highest amplification efficiency and specificity was found by I-PEP, especially when microdissected cells were investigated. I-PEP-PCR was successful in preamplification of DNA from 30 formalin-fixed paraffin-embedded cells. In contrast, unmodified PEP-PCR failed to preamplify DNA of 100 or less cells and showed reproducible results only with more than 500 cells. Furthermore, I-PEP-PCR is also more efficient in amplification of fragments greater than 500 bp as compared to unmodified PEP-PCR (100% vs. 20% amplification rate in multiple samples of five SW480 cells, sorted by flow cytometry). In addition, I-PEP-PCR enables mutation analysis by conventional sequencing as we have not found any artificial I-PEP-induced mutation in more than 4000 sequenced bases of p53 exon 7 and 8 in 10 breast cancers. I-PEP revealed the same mutations as detected in non-preamplified samples. Applications of I-PEP in LOH and sequencing analyses of microdissected preneoplastic lesions in the pancreas and urinary bladder as well as in disseminated tumor cells of the bone marrow will be presented.

**#875 A molecular cytogenetic analysis of 7q31 in prostate cancer.** Qian, J., Lee, H.K., Huang, H., Hirasawa, K., Bostwick, D.G., Proffitt, J., Wilber, K., Lieber, M.M., Liu, W., Smith, D.I., and Jenkins, R.B. Mayo Clinic, Rochester, MN 55905; Vysis Inc., Downers Grove, IL 60515.

To better understand the chromosome 7 alterations in prostate cancer, we undertook a molecular cytogenetic study of 25 prostate specimens using fluorescence *in situ* hybridization (FISH) with DNA probes for the chromosome 7 centromere and for 5 loci mapped to 7q31 (D7S523, D7S486, D7S522, D7S480, D7S490) and 1 locus at 7q11.23 (ELN). Six tumors had no apparent anomaly for any chromosome 7 probe. Nine tumors showed apparent simple gain of a whole chromosome 7, whereas one tumor had apparent simple loss of a whole chromosome 7. Four tumors had gain of the chromosome 7 centromere and additional overrepresentation of the 7 q-arm. One tumor had overrepresentation of 7q31 without any apparent anomaly of the chromosome 7 centromere; and one tumor had apparent loss of the chromosome 7 centromere with no apparent anomaly of the 7 q-arm. Three tumors had gain of the chromosome 7 centromere and loss of the 7q31 region. Gain of 7q31 was strongly correlated with tumor Gleason score. Multiplex PCR studies of these specimens supported these FISH observations. Mutation screening and DNA sequencing of the MET gene, which is mapped to 7q31, revealed only the presence of simple sequence polymorphisms but no apparent acquired disease-associated mutations. FISH analysis of metaphases from an aphidicolin-induced, chromosome 7 only, somatic cell hybrid demonstrated that the DNA probe for D7S522 spans the common fragile site FRA7G at 7q31. Our data indicate that the 7 q-arm, and particularly the 7q31 region, is genetically unstable in prostate cancer, and some of the gene dosage differences observed may be due to fragility at FRA7G.

**#876 Altered expression of the suppressor gene PML in human liver and lung cancer.** John YH Chan<sup>1</sup>, Wai Chin<sup>1</sup>, Louis Chow<sup>2</sup>, Choong-Tsiek Liew<sup>2</sup>, Sing-Fai Leung<sup>1</sup>, Anthony Yim<sup>3</sup>, Kun-Sang Chang<sup>4</sup> and Philip J Johnson<sup>1</sup>. <sup>1</sup>Clinical Oncology, <sup>2</sup>A and C Pathology, <sup>3</sup>Surgery, YK Pao Centre for Cancer, Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong, <sup>4</sup>Laboratory Medicine, UT MD Anderson Cancer Center, Houston TX, 77030.

The promyelocytic leukemia (PML) gene, which encodes a growth suppressor, was first identified at the chromosomal t(15;17) translocation break point of acute promyelocytic leukemia. To elucidate if PML may be involved in other neoplasias, the expression of PML was analyzed by immunohistochemical staining in small cell lung cancer (SCLC), adenocarcinoma (ADC), squamous cell carcinoma (SCC) and hepatocellular carcinoma (HCC). PML expression was completely suppressed in all of the SCLC examined, whilst enhanced and/or moderate expressions were found in HCC, ADC and SCC. In HCC and SCC, PML was expressed most strongly in the cancer cells at the periphery of the tumors and in the surrounding non-tumor cells, while the expression was progressively decrease towards the tumor-center. Double staining with Ki67 and Kp1 showed that this decrease is not necessarily associated with decreased proliferation but may be related to altered transformed phenotypes. The specific inactivation of PML in SCLC indicate that this alteration may be an obligatory step in the tumorigenesis of SCLC.

**#877 Computer assisted cytogenetic analysis reveals differences between malignant schwannomas of patients with and without neurofibromatosis.** Plaat, B.E.C., Mastik, M.F., Molenaar, W.M., Van den Berg, E. Depts. of Pathology and Medical Genetics, University of Groningen, Groningen, The Netherlands.

**Introduction:** Cytogenetic studies of malignant schwannomas in small groups of patients with and without neurofibromatosis (NF1) revealed complex karyotypes in which no consistent chromosomal changes were detected. To allow direct comparison of the complex karyotypes of 28 malignant schwannomas (16 NF1 and 12 non-NF1 patients) obtained from the literature and own data (9 cases), a database was constructed using Dbase V-Windows. The breakpoints,